THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME VI NOVEMBER, 1930 NUMBER 6

RETICULIN *

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Since the fundamental work of Mall,[1] histologists have busied themselves much with reticulin. Interest in this form of connective tissue increased when silver impregnation provided the means for more precise morphological study. One fact particularly attracted attention; in successful preparations, reticulin stains black while collagen bundles stain yellow, thereby fortifying Mall's conclusion that reticulin, yielding no gelatin on boiling, is a substance different from collagen. Many authors now designate it as the argyrophil reticulum, contrasting it with the collagen bundles, which are not believed to be arranged in a network.

Partisans of the unitary hypothesis, Mallory and Parker[2] among them, insist that reticulin does not differ essentially from the collagenous framework with which its substance is continuous. If it colors black with silver while the collagen bundles stain yellow, this is because its finer fibers are more easily penetrated by the silver; moreover, with truly elective methods, the staining affinities of the two substances are identical.

Recently, however, by ingenious experiments, Foot[3] has sought to establish the reason for the argyrophilia. He attributes it to a special substance which impregnates the reticulin and which can be extracted by sodium hydrate.

In this paper, we shall describe briefly our observations of the structure of several types of reticulin. We shall then study some new facts relating to the physiochemical constitution of the fibrils of the collagen framework in general, which seem to us to support the unitary hypothesis.

* Received for publication July 23, 1930.

Reticulin of the Areolar Tissue

With a hypodermic syringe, 1 or 2 cc. of Locke's solution are injected into the subcutaneous areolar tissue of the abdomen of a rat. There is thus produced a well defined, edematous tumor in which the connective tissue elements, normally compressed so closely that it is impossible to study them, are separated uniformly so that their arrangement can be examined in all of its details.[4] This valuable technique is due to Ranvier.

The edematous tumor looks like a transparent jelly. A thin slice cut with the curved scissors and immersed in Locke's solution holds its shape. It may be mounted between slide and cover glass and examined with the ultramicroscope, which reveals perfectly the collagen bundles and the reticulin of which this tissue consists.[5] The entire thickness of the abdominal wall may be cut out around the tumor, fixed in Zenker's or Helly's fluid and embedded in paraffin, in order to obtain thin sections for staining with Mallory's method; or it may be embedded in celloidin, thick sections impregnated with silver by the usual methods and mounted in balsam, after having dissolved and removed the celloidin.

The latter preparations are particularly instructive (Fig. 1). Despite their thickness, which may exceed 50 microns, they are perfectly transparent. Changing the focus enables us to follow the fibers over long stretches. The coarse collagen bundles are ribbon-like, wavy along their borders as along their surfaces and are pale yellow. The opaque black reticulin extends out into the interstices so that all of its fibrils and meshes are seen distinctly. Some parts form networks of two dimensions which, at their borders, are continuous with networks of three dimensions. In all of these networks the elements are uniformly separated by the injected fluid so that the relations between them are not altered; nowhere is there the least tearing of tissue. The difference from the normal state is of the same order as the difference between lace which is wet and crumpled and the same lace spread out, each thread being detached on an optically empty ground. Moreover, if the edematous tumor is left to follow its natural course in the living animal, it will disappear by itself in a short time and there is nothing, either to the naked eye or on microscopic examination, to reveal its former location; no lesion remains.

On examining preparations of the edematous tumor we see the coarser bundles of collagen ramifying in more slender branches, and these in their turn ramifying in finer and finer fibrils, ending in reticulin. Like the reticulin, the large bundles are arranged in networks. The only difference is that the meshes of the reticulin are so small that they are seen easily in thin sections, whereas the collagen bundles form a network of such large meshes that they cannot be followed in their entire extent even in thick sections; but between the two are all intermediaries.

In sections stained by the selective methods of Mallory and Van Gieson we may establish another fact: all of the collagen fibers and fibrils, when standing alone, stain alike, the intensity depending solely on their thickness. Comparison of these preparations with those of the same tissue fixed in the natural state, where the elements are compressed one against the other, shows that all that which has been believed to be amorphous or fundamental substance is nothing but compact reticulin;[6] but the degree of condensation of the reticulin fibrils, when compressed one against the other, is less than that of the fibrils in the interior of the collagen bundles. Hence it comes about that the so-called amorphous substance appears to be stained less deeply than the bundles. On the supposition that this substance constitutes a primitive phase in the development of the bundles it has been called precollagen, and diminished colorability is given as one of its characteristics. This distinction has no serious basis either in anatomy or in embryology. It is obvious that in embryonic development the more slender forms will appear first, to increase later at all points where there is no reason for them to remain slender; but there is no evidence of a chemical evolution in the substance of the framework except in certain tissues such as bone. In reality, the entire collagenous framework of the areolar tissue is made of one and the same substance which is disposed in fibrils, either free or united in bundles, without any visible trace of amorphous substance, at least not in mammals.

Finally, we should note that in areolar tissue the fibroblasts, even though regularly distributed, do not provoke any change in the framework of their immediate neighborhood; they have no appointed places; they are scattered at random and take the form that is imposed on them by the space which they occupy. In a word, they are modelled by the framework, the structure of which they do not control.

Reticulin of Nerve, Muscle and Fat

Although the noble elements of these tissues are as different from one another as it is possible to be, their respective reticulins are constructed on exactly the same principle; and this principle is precisely the opposite of that which we have described as governing the relations between the fibroblasts and the framework of the areolar tissue. Instead of submitting passively to the influence of the framework and allowing itself to be modelled by it, as do the fibroblasts, each fat cell, each nerve fiber and each muscle fiber envelopes itself in an extremely delicate and close-meshed reticulum, which constitutes an individual sheath closely applied to it.

These close-meshed, reticulated sheaths spring from branches of fine collagen fibers of a much coarser network that lies between the noble elements, so that the sheaths form a continuous whole even though each sheath is distinctly individualized.

In the *nerve*, the ensemble of the framework forms the endoneurium, the structure of which was described but incompletely by the earlier authors who studied it with silver. Plenk [7] first sketched the details, but these were studied more precisely by Laidlaw [8, 9, 10] with the aid of his excellent method of silver impregnation.

In the *muscle*, the perimysium is constructed on a similar type. It has long been known; we shall not dwell on it further.

We ourselves have studied the *adipose tissue* in thick frozen sections.[11] This technique furnishes preparations that are imperfect as a whole, but in which certain points present images more demonstrative than thin paraffin sections. Fig. 2 represents one of these points. It shows the relations between the protoplasmic wall of the fat cell and the reticulated envelope, and between this individual sheath of the fat cell and the interstitial framework common to the tissue.

In closing the discussion of reticulin under this category, we should point out that in both nerve and muscle the reticulin contains fibroblasts which, as elsewhere, lodge as best they may; whereas each of the noble elements has its own habitation, carefully constructed. In the fat, however, there are no fibroblasts in the interior of the lobules; besides the fat cells, the lobules contain nothing but mast cells and a network of capillaries without any accompanying connective tissue. In order to find fibroblasts, one must search as far as the

delicate connective tissue envelope of the fat lobules. What, then, is the cell that is responsible for the formation of the reticulin?

Reticulin of the Liver and of the Spleen

The anatomical arrangement of the reticulated framework of these two organs is too well known for us to describe it anew. We shall say simply that in the liver the reticulin lies in the only free space, the space between the columns of liver cells and the capillaries. In consequence, its orientation may be attributed as well to the one as to the other of these two elements. As for the fibroblasts, they remain cantonned in the portal and subhepatic spaces at such a distance as to render very doubtful their rôle in the fabrication of the framework.

In the spleen there is an obvious connection between the disposition of the reticulated framework and that of the cells of the splenic parenchyma. In the malphigian bodies the lymphocytes influence the network rather as a group. In contrast, the cells of the pulp are individually enshrouded in the meshes of a very regular network which is moulded on them. Its fibers present a remarkable feature; they are finely and uniformly wavy, so much so that they resemble spirochetes, this form being doubtless connected with the great variations in volume of the spleen under physiological conditions. Here, too, the cells peculiar to the reticulum, which represent fibroblasts, have no influence whatever on the orientation of the framework, this being governed entirely by the cells of the parenchyma.

These organs especially are favorable objects for the study of the changes made in their substance by the different treatments that may be applied. Foot has the merit of commencing studies in this field. We have merely sought to verify his results, but our conclusions differ very much from his.

In the first place, we find ourselves differing from our colleague on certain points of technique. This question will be discussed in another paper. We shall say here that, contrary to Foot's statement, the reticulin can be impregnated perfectly in black and without difficulty in frozen sections of fresh organs, with no other fixation than that resulting from the passage through the ammoniacal silver bath.

As to the results obtained on boiling the spleen, our experiments confirm those of Foot in a general way, but we do not draw the same

conclusions from them. The change in fuchsinophilia with Van Gieson's method seems to us to be correlated with the progressive change and destruction of the framework, which necessarily begins in the most delicate portions, that is to say, the reticulin of the pulp. That of the trabeculae resists longer because it is more coarse. The connective tissue bundles of the capsule are quite well stained after boiling for half an hour. This is easily understood. In order to prevent their staining by acid fuchsin, the collagen must not only be transformed completely into gelatin but the gelatin must be dissolved and dispersed, which occurs but slowly. In reality, neutral gelatin stains red with Van Gieson, and not yellow as Foot states. It may lose its fuchsinophilia, it is true, but for this change to take place it is necessary that the pH fall below the isoelectric point. No doubt the red staining of the gelatin is less intense than that of the collagen fibers, but this is because it contains more water.

Like Foot, we have found that reticulin, after boiling, is still colored black by silver, but the integrity of the network persists much longer in the liver than in the spleen. After fifteen minutes of active boiling the reticulin of the liver appears relatively well preserved in form and it colors black; but its meshes are smaller, due to the shrinking of the hepatic cells and to the shortening of the fibrils, which contract under the influence of heat exactly like connective tissue fibers. Boiling must be prolonged for half an hour to effect a profound change in the reticulin, in its structure but not in its staining. It swells considerably, trebling the diameter of the fibrils, and these segment into small discs, giving them a transversely striated appearance (Figs. 3, 4, 5). In the spleen, on the contrary, this change occurs as early as the fifth minute of boiling. The reticulum of the pulp has become very incomplete, visible only here and there, but that which remains still stains black. Like the reticulin of the liver, the fibers of the trabeculae and those of the capsule are striated transversely because of their disintegration into discs formed of granules that reduce the silver. After boiling for fifteen minutes or even half an hour, this aspect scarcely changes, but in place of the network fragmented into granules at the beginning of boiling and now having disappeared in form, there remains the débris of its substance which still stains with silver. The splenic reticulin, then, is less resistant to heat than the hepatic reticulin, but despite the changes undergone, the substance of the one, as of the other, con-

tinues to color black with silver as long as it is not completely disintegrated.

In short, staining by silver and by acid fuchsin both show progressive alteration of the framework, ending in its destruction; but silver enables us to see the débris of the reticulin as long as it exists, whereas fuchsin does not. Is there anything surprising in this? The Van Gieson stain reveals almost nothing of the intact framework of the liver or of the splenic pulp as long as the cells of these organs remain in place; and the staining power of the fuchsin on reticulin diminishes necessarily when the proteid matter of the fibrils swells and is diluted by the absorption of water in boiling. The swelling may be estimated as at least twenty times the original volume and the intensity of the staining per unit volume must be decreased in the same proportion. In coloring by metallic impregnation, on the contrary, there is no necessary relation between the volume of the colloidal silver precipitated and the quantity of the proteid molecules. After boiling, the swelling of the fibers, augmenting their permeability, may favor the accumulation of the metal and produce the opacity despite the absorption of water. Thus the contrast described by Foot between the conservation of the argyrophilia and the diminution of the fuchsinophilia in boiled reticulin finds a natural explanation and there is no need of any hypothesis.

Furthermore, it is obvious that boiling, which fragments the reticulin, changes its substance much more than does sodium hydrate which, acting for twenty-four hours, causes no morphological change in the reticulum, does not destroy the fuchsinophilia, as Foot has proved, or its argyrophilia, as we shall show. In fact, concerning the disappearance of the argyrophilia after treatment with sodium hydrate, we are obliged to contradict Foot's statements.

Slices of liver and spleen of dogs, about 5 or 6 mm. thick, were immersed for twenty-four hours in normal sodium hydrate (4 per cent), washed thoroughly until neutral, fixed in Helly's fluid and frozen sections made. The reticulin impregnates black without the slightest difficulty.

In another experiment, small pieces of dog's liver, 2 mm. thick, were suspended from tiny glass hooks which served to transport them to the different fluids without exerting the least mechanical action on them. After twenty-four hours in the sodium hydrate, where they became white and very soft, some were immersed in

N:500 HCl, others in pure water. In these fluids, they expelled almost the entire quantity of their protoplasm in the form of white clouds, falling slowly to the bottom of the receptacle. After complete neutralization they were fixed in formol, or Zenker's or Helly's fluid, embedded in paraffin and cut in thin sections; with silver, the reticulin colored a perfect black (Fig. 6). The argyrophilia of the reticulin, then, cannot be due to an argyrophil substance impregnating the fibrils and susceptible of removal or destruction by the action of 4 per cent sodium hydrate for twenty-four hours.

Stained by Van Gieson's or by Mallory's method, these sections, almost completely freed from cells, show the reticulin stained selectively with perfect clearness, whereas in ordinary sections its existence would scarcely be suspected. It is well known that an analogous procedure, more appropriate for histological research, was used by Mall, who resorted to pancreatin to rid the tissue of the confusing cells and to reveal the reticulin in an excellent manner at a time when silver impregnation was unknown.

The Rôle of Physical Properties in Silver Technique

The achievements as well as the defects of silver technique depend on this, that they rest on physical properties rather than on chemical affinities, as we shall now show. In a section of connective tissue colored with silver, the large bundles are yellow, the smaller bundles are darker as they become smaller and the finer fibrils are black. It is said currently that the parts of the connective tissue network that stain black are argyrophil, which would imply that the parts colored yellow are argyrophobe; but this selectivity of the two colors is far from being invariable. Varying with the fixative and the slightest details of technique, the black is seen to encroach more and more on the yellow, and indeed it is not difficult to stain all black. Thus Laidlaw's method [8, 9, 10] for the peripheral nerves colors the pia mater exactly like the individual reticulated sheaths of the peripheral nerve fibers. The color of the reduced silver, then, so far as it concerns the elements of the connective tissue framework, is a relative and not an absolute property.

Coloring by silver depends on the formation of a colloidal sol which fixes itself on the constituents of the tissues, themselves also

in the colloidal state. In the first place, we know that the color and opacity of a metallic sol depend on the dispersion of the metal, that is, the dimensions of its particles, much more than on the quantity of metal in suspension. In the second place, hydrophil colloids, that is to say, albumins, and in consequence the substances composing the connective tissue framework, have a permeability which varies much more according to their hydration than according to their chemical constitution. The composition of the baths, their concentration, the proportion between the silver salt and the reducer, all play a capital part in the degree of dispersion of the colloidal silver which is produced in the course of the reduction.

If these factors should operate in a homogeneous medium, perfectly permeable to all the substances employed, the resulting color would be uniform; but collagen, even supposing it to have an invariable chemical composition, is far from being homogeneous and its permeability is not invariable. Being itself colloidal, its dispersion may vary from one point to another; and it is arranged in fibrils which are sometimes free and merely alongside of one another, sometimes massed in dense bundles. The fibrils of a connective tissue bundle are not associated accidentally and temporarily. The bundle is a stable anatomical unit which does not dissociate in the edematous tumor when all the fibrils of the reticulin separate one from another. What is the cause of this stability? We do not know exactly but, as we shall show presently, there are reasons for believing that around each bundle there is a membrane, too thin to be demonstrated and yet capable of modifying the permeability of the whole bundle; and that, in the interior, the fibrils are more or less agglutinated, with what substance we are unable to see. These are conditions which differ physically very much from those in which the fibrils of reticulin find themselves.

Permeability is a very important factor. It affects the concentration of the penetrating reagents, probably more that of the reducer than that of the silver. In Cajal's method, the periphery of the piece is black, even if it has been cut before immersion in the reducer. Toward the center it is paler and paler yellow, more especially because the reducer arrives there more and more diluted. Moreover, in the zone where the general color is yellow, the large axons are yellow while the small ones are black. This is exactly what happens among connective tissue fibers colored with silver.

The penetration of substances dissolved in a heterogeneous colloidal medium is an extremely complex phenomenon. At each passage from one phase to another, there occur reactions which modify their distribution on the one side or the other of what are known as the interfaces, and these reactions may influence the reduction of silver in the silver techniques, which would explain the accumulation of colloidal precipitate in these interfaces. As we shall see presently, this is easily demonstrated. There are other properties of the interfaces, however, which perhaps play a still more important rôle in the process under consideration.

We would speak of the phenomena connected with the radius of curvature and consequently the surface tension, which increases rapidly as the dimensions of the object diminish. Quincke has calculated the conditions of equilibrium in cells of various dimensions, all other conditions being equal. For spheres of radii of 10 microns, 1, and 0.08 micron, the interior pressure must exceed the external pressure by 0.046, 0.46 and 5.75 atmospheres respectively. With collagen fibers, which have no semipermeable membrane, it is not the osmotic pressure that intervenes, as with cells, but the pressure of imbibition. Practically it amounts to the same thing, for, in the swelling of colloids, the water is absorbed much more in proportion than the salts.

To the differences in internal pressure are added necessarily differences in the electric phenomena in the interfaces; for these phenomena also depend on the size of the particle. We know the considerable rôle of electricity in colloidal phenomena and consequently in the methods of silver impregnation.

In support of what we have just said on the importance of the interfaces, we shall present two orders of facts: (1) the manner in which fibrin behaves toward silver according to the medium in which the fibrils occur; and (2) the constitution of the silver sol in the connective tissue preparations as revealed by the ultramicroscope.

Conditions under Which Fibrin Colors Black with Silver

Fibrin formed in the tissues passes for non-argyrophil. We have not yet sufficiently studied this question, which the variability of fibrin makes very complex, to have a personal opinion on this point. This much is certain, that the thin clot of citrated plasma formed on

a slide by adding a drop of a solution of calcium chloride to a drop of plasma, washed to remove the albumin of the serum and fixed in Helly's fluid, impregnates an opaque black just as well as does reticulin (Fig. 7). However, if we use the spontaneous clot removed from a centrifuge tube in which it has formed, wash it superficially, fix and embed in paraffin, the result is quite different. At the periphery, in the thin layer that has been affected by the washing, the fibrils impregnate black, the better as they are nearer the surface; in the center is seen nothing but granular matter uniformly colored brown, without the least trace of fibrils. Nevertheless fibrils exist at the center as well as at the periphery, for phosphotungstic acid hematoxylin brings them out very clearly. A change in the medium outside of the interface, then, suffices for the material inside of it, the fibrin, to manifest an argyrophilia that had been completely masked.

Ultramicroscopic Study of Silvered Preparations

The ultramicroscope, the instrument *par excellence* for the study of colloids, enables us to see interesting aspects of silvered preparations, both those stained by Cajal's method and those treated by the techniques derived from that of Bielschowsky. In sections mounted in balsam, all luminous phenomena due to the hydrophil colloids of the tissues vanish, leaving visible those belonging properly to the colloidal substances introduced by the fixative or by the staining fluid, and especially to the colloidal silver that is fixed on the anatomical elements. In this way we may study the metallic sol, recognize the degree of fineness of the granules, which varies according to the territories where deposited, and learn the peculiarities of its elective distribution.

With transverse illumination of a preparation colored with silver, the reticulin is brilliantly illuminated in white, sometimes also in yellow, in a dark field. The collagen bundles, on the contrary, when they have been colored yellow in the preparation are illuminated diffusely, though permitting their fibrils to be seen more or less; but when the light is regulated carefully, their borders give a bright reflection of yellow tint. It is obvious that their surface is mirror-like (Fig. 8).

In a fine reticulin, such as that of the edematous tumor or of the splenic pulp, each filament appears as a simple brilliant line. In

coarser reticulin such as that of the liver of the larger animals, the fibrils are bordered on each side by a bright line but the central portion remains dark.

With higher magnification all of these lines appear finely granular, even when the impregnation is very pure and when the filaments appear smooth by transmitted light. It is obvious that this appearance is due to a multitude of tiny reflecting surfaces. In the yellow areas the silver granule is smaller, often invisible; the aspect is that of a submicroscopic sol and the reflection by the surface of the collagen bundle is similar to that of a smooth surface. The yellow, transparent silver, then, is more highly dispersed than the opaque silver which appears black by transmitted light because of its opacity.

The blackening of the fibers is partly due to a thick deposit of silver on their surface, for their diameter seems greater when colored with silver than by other methods. Moreover, on examining coarse fibers, those of the liver for instance, with high magnification and transmitted light, we see that their center is not absolutely opaque. If the color were due exclusively to a uniform impregnation of the entire thickness of the substance, such an appearance would not be produced. A metallic deposit on the surface of a filament is the result of physical attraction and not of chemical combination with the substance of the filament.

On the surface of the yellow collagen bundles, by transmitted light, we see nothing indicating a deposit of silver. This deposit exists nevertheless, for it reflects in the ultramicroscope, but it is too thin and too transparent to be seen by direct illumination. It is even possible that it is this thin veil of silver on the surface of the yellow objects that gives to the images in sections stained by Cajal's method, even when they are very thick, that perfect definition of the finest details, often noticed, which no other technique has attained.

In the course of a silver impregnation we may concede two successive phases: (1) colloidal silver is produced as a chemical phenomenon in the reagents; (2) it localizes and fixes itself by a phenomenon of election which rests on the physical properties of the tissues. Obviously, the physical properties of a body depend on its chemical constitution but only in a certain measure. There is no question that different bodies may realize equivalent physical conditions and, on the contrary, the same body, according to circumstances, may acquire different physical properties. It follows that various sub-

stances may impregnate black while the same substance, of the collagen group, may impregnate either in black or in yellow according to circumstances. The argument in favor of the duality of substance, based on the different color taken by reticulin and by the collagen bundles, is of no value.

Physicochemical Constitution of the Collagen Framework

What we have just said does not mean that collagen is identical in all vertebrates, nor that in animals of the same species it is identical in all parts of the connective tissue framework; it means simply that histological methods are impotent to reveal the variations which logically one might suppose to be present.

Like all albumins, collagen is a polypeptid. We know that these substances vary infinitely according to the number and kind of their constituent amino-acids, the manner of union of the radicles, the polymerization of the molecules. Among the albumins there are classes characterized by certain primordial properties; among these classes there are groups possessing secondary properties in common, and so on as far as the ultimate variety which differs from its neighbor only in an insignificant detail, and which cannot be distinguished with certainty by any means at our disposal.

A priori, it is quite probable that collagen has not a uniform composition in the whole extent of the connective tissue framework, and it is certain that it varies from one species of animal to another. In the first place, what is collagen? We have reasons for believing that it is a complex formed by a certain kind of protein with neutral salts of metals that exist in the animal economy. Here already is a cause of variability, for these salts are many and it is not unreasonable to suppose that each connective tissue fiber, whether of reticulin or of a collagen bundle, may consist of a mixture of molecules differing one from another by the salt which they contain, the proportions of the mixture differing from one organ to another. The albumin may vary also.

We know that the connective tissue substance appears between the cells, in contact with them but not by the exfoliation of a so-call edexoplasm which has been supposed to detach itself from their substance. Maximow's observations in tissue cultures, in which he

succeeded in obtaining a connective tissue framework, have confirmed this absolutely,[12] and it had already been demonstrated by the methods of pure histology. What cells have the power of invoking the appearance of a connective tissue framework? Fibroblasts inhabit almost the entire extent of the framework, and it is quite natural to believe that they play the principal part in its formation; but we must not be too exclusive. In adult tissue, we have already shown the intimate relations existing between reticulin and various cells in territories where fibroblasts exist and also where they do not exist. This is much more obvious in the embryo where certain dispositions of the framework around the notochord (von Ebner, Klaatsch) and in the cornea (Kessler) cannot be explained reasonably if one would reserve to the fibroblasts alone the privilege of fabricating the interstitial substance. In reality, many kinds of cells are capable of collaborating in the construction of the connective tissue edifice. What reasons have we for believing, therefore, that all of these collagens of diverse origins are identical?

Let us leave to one side the question of the multiple origins of collagen which will occupy us later on; let us not insist on those instances where the connective tissue plays an accessory rôle of interstitial framework for the noble elements of the viscera, of the muscles, of the nerves or merely of the subcutaneous fat. Let us limit ourselves to the forms in which of itself it constitutes a part of the organism and where as fixed cells it contains only fibroblasts, the derma, the subcutaneous areolar tissue and the tendons. Among these different forms of collagenous tissue we find considerable differences, not only of texture but also in the dimensions and grouping of the fibrils, the transparency or, on the contrary, the reflecting property of the bundles, their aptitude to swell—in a word, physical variations so great that we must be tempted to suppose them bound to chemical variations in spite of the uniformity of their staining affinities.

These are the *tissue variations* of the collagen framework which end in the formation of different connective tissues; but each one of these tissues, in its turn, may not be identical in all regions of the organisms. This fact is obvious for the derma and we shall give presently a remarkable example for the tendon tissue. There are, then, *regional variations* which superpose themselves on the tissue variations.

Must we seek then among the fibroblasts for special varieties endowed with different potentialities as the first cause of all these anatomical, physical and probably also chemical variations of the collagen framework? Scarcely. When dead tendons are grafted in areolar tissue, the graft attracts the fibroblasts from the surrounding tissues, and at the same time that it is vacularized it is repeopled completely, to the point that it resumes definitely the properties of living tissue. The fibroblasts of the areolar tissue change their form when they penetrate the tendinous framework and assume immediately all the characteristics of tendon fibroblasts.[13, 14, 15] In our opinion, this experiment shows that even those varieties of fibroblasts that are most dissimilar in appearance possess in reality the same powers. Hence we are led to consider the different forms of collagenous framework as conditioned essentially by the place where they develop in the organism and by the totality of the interrelations which govern in the territories which they occupy.

As far as collagen is concerned we can distinguish these diverse substances produced by cells of the same species only by differences in certain physical properties; but there exist other and more favorable substances where it is easy to present evidence of chemical differences. As an example we shall take the regional variations of a substance entirely different from the albumins, but which lends itself particularly well to the demonstration. The subcutaneous white adipose tissue is everywhere similar, in appearance at least, but already the microscope shows that the fat cells, otherwise specifically identical, have different dimensions according to the different regions of the body. In Delphinus tursio, Margaillan [16] has shown that, even though the contents of the fat cells everywhere consists of oil, it differs none the less in chemical composition from one territory to another. These chemical territories are more or less extensive (in the head, for instance, there are three very small ones) but their anatomical delimitation is invariable. Moreover, the different chemical territories are not scattered in a disorderly manner; the variations observed in the chemical composition of the oil follow a systematic order in the entire extent of the subcutaneous fat. In a word, there is a chemical architecture of the fat comparable to the cyto-architectonic and the myelo-architectonic of the cerebral cortex.

Chemical variations of this kind, which are established with certainty for the fat and which probably exist also in the connective

tissue, present great interest from the general point of view. They show that the specific activity of homologous cells is everywhere subject to the influence of a regulation of a superior order. It is the entire organism, an energy system, coördinated and equilibrated in all of its parts, which controls the quality as well as the quantity of the substances elaborated by each one of its elements: it matters little whether these products are intracellular or intercellular. However, by its very generality, this principle ceases to be applicable when we are searching for a specific difference between reticulin and the collagenous substance which are mixed intimately in the same tissue.

In fact, it is probable that reticulin is not identical everywhere. We have shown that the reticulin of the spleen, for example, behaves differently from that of the liver under the influence of heat. Does this result from different properties or merely from greater fragility due to the delicacy of the splenic reticulum? Or from the action of substances expelled from the cells by the heat? Of this, we can assert nothing.

Since Mall's time, it has been believed that the great characteristic distinction between collagen and reticulin is the yielding or non-yielding of gelatin. We have tried to verify Mall's statement that the action of pancreatin usually prevents the formation of gelatin and have found that tendons digested for twenty-four hours in the incubator, in a solution of pancreatin of Mall's formula and freed from all cellular elements, yield gelatin on boiling as easily as fresh tendons. Moreover, in those organs where the authors find no gelatin, it may be that the gelatin is merely made soluble in the cold and consequently prevented from jelling by the action of substances coming from the parenchymatous cells, the mass of which is infinitely greater than that of the reticulin, and that for this reason the presence of gelatin was overlooked.

Suppose that we should succeed in eliminating this error and demonstrate to a certainty that, as far as the production of gelatin is concerned, the framework of a viscus is different from that of a given connective tissue; still this does not suffice to establish a specific difference between the two substances, *collagen* on the one hand and *reticulin* on the other. To reach this conclusion, it is necessary to prove a difference between the chemical constitution of collagen and reticulin in the same tissue, the areolar tissue, for instance.

We have reason to believe that the substance of the reticulin of areolar tissue, which is very abundant, does not differ from that of the collagen bundles. If, after having spread on a slide a frozen section of the unfixed edematous tumor and applied a cover-glass, the preparation be heated along a narrow line by an electric current passing through a wire applied to the under surface of the slide, the section is seen to divide suddenly into two parts as soon as the temperature reaches a certain degree. The reticulin and the collagen bundles liquefy at the same moment.

To summarize, we are led to conclude that, extensive as we may suppose the tissue and regional variations of the chemical constitution of the different elements of the connective tissue framework to be (the elastic fibers excepted), there is not at present any reason for believing that these differences can furnish a means of separating two types, systematically distinct although intimately mingled.

The Artificial Production of Collagen Fibers in Feltworks, or in a Reticulum with Closed Meshes

Under the microscope, with transverse illumination, it is easy to watch the formation of an artificial fibrous clot of collagen. The fibrils appear in the form of extremely fine filaments, scarcely perceptible, which, in a few minutes, elongate, thicken and become luminous at the same time that their structure seems to become more intricate.

To observe these phenomena, it suffices to treat the tendons of a rat's tail for twenty-four hours with a dilute acid, acetic acid 1:25,000, for instance. The tendons are obtained by beginning at the tip of the tail and tearing off pieces 1 cm. long. One tail yields about 70 cg. of tendons, which are placed in a dish containing 50 cc. of the acetic acid solution, taking care to disperse them well throughout the fluid. If, after half an hour, there is not much swelling, change for fresh fluid. In twenty-four hours the tendons will be much swollen and very fragile. The solution is filtered through several thicknesses of gauze. It is limpid, slightly viscid, and contains a little more than 1:1000 of dissolved substance.

An opalescent fibrous clot can be produced in a test tube by adding to this fluid solutions of neutral salts of various concentrations (NaCl from 4 to 20 per mille); or the acid may be removed by dialyz-

ing in a collodion sac. The liquid then takes the form of a limpid jelly which melts irreversibly about 50°C. If, instead of heating this jelly, it is acidified again, it is redissolved and becomes again capable of forming a fibrous clot by the action of neutral salts.

The dissolved substance is the albumin of the collagen, its salts having been removed by the reversible action of the acid. If the acid is removed by dialysis and the dissolved salts at the same time, the albumin jells; but if, without removing the acid, a sufficient proportion of salt is added, there is produced a mass action, the result of which is the reconstitution of the complex albumin plus neutral salt, and the reappearance of the collagen in its typical form, which is fibrillar.

These phenomena were long studied by one of us in a series of notes.[17, 18, 19] We shall repeat here only what can contribute to the discussion of reticulin.

In order to observe the formation of the clot under the microscope, two thin, narrow streaks of paraffin should be traced with a hot iron along the edges of a slide. A small drop of the tendon solution is placed in the middle and covered with a cover glass, which is cemented along the borders that rest on the paraffin. The drop, flattened between the two glasses, should be free all around its margin. The free space is then filled with a 1 per cent solution of NaCl. The drop disappears but soon there is formed in its place the reconstituted clot of collagen which can be studied with the ultramicroscope. It may be fixed also in neutral formol, in Bouin, Zenker's or Helly's fluid after washing in water to remove all traces of acid; then separate the two glasses and treat the layer of clot adherent to each of them like any mounted section.

It is then seen that the clot is formed of fibrils, the thickness of which varies in different preparations. These fibrils are simple and arranged in feltworks, or they may ramify and anastomose with one another in the form of networks with closed meshes which may strangely resemble reticulin (Fig. 9).

In favorable preparations, with the aid of the ultramicroscope, details may be seen in the thickness of the coarser fibers, aspects that indicate the endogenous formation of secondary fibrils inside of the primitive fibrils, all crossing and growing more intricate by intussusception. In our opinion, this throws light on the mode of formation

of collagen bundles. We may thus explain the relations of the fibers to one another in these bundles and also their enveloping membrane, the existence of which we have been led to suppose from the mirror that forms on the surface of the silvered sections.

We shall call the substance of this artificial clot Collagen A. In polarized light, its fibrils have the same birefringence as those of natural collagen. Like the latter, they may be fixed and stained by selective methods. They take the anilin blue of Mallory, the acid fuchsin of Van Gieson; they stain red with phosphotungstic acid hematoxylin. Finally, although their substance was derived from tendon, they are as "argyrophil" as the fibers of reticulin itself, for they impregnate easily in opaque black. Collagen A is certainly very close to the natural collagen. It differs only in its greater solubility in dilute acids, probably due to a slight polymerization.

Only the tendons of the rat's tail give Collagen A. The other tendons swell without dissolving. However, another substance, differing from Collagen A but also coagulable in fibrils by the action of neutral salts, may be extracted from the tendons of the rabbit and the dog. We shall designate this substance Collagen B. The technique is as follows: The tendons are left to swell for twenty-four hours in normal solution of sodium hydrate (4 per cent). They are then washed to the point of neutrality and treated with dilute acid, as already described. There results a fluid which, treated as before, yields a fibrous clot. This clot differs from Collagen A in that its fibers are finer, tortuous, always ramified and anastomosed in closed networks of peculiar aspect. Moreover the clot is fragile, but when treated by the selective histological methods it stains exactly like the clot of Collagen A and it also impregnates perfectly black with silver (Fig. 10). Here then is a substance which, after having resisted sodium hydrate, is yielded by the tendon when treated with dilute acids and which is no less argyrophil than reticulin itself.

These experiments show that it is useless to try to distinguish reticulin chemically from collagen by the color that it takes with silver. They show also how much homologous substances, though colored alike by the same technique, may differ when submitted to other methods of investigation. Under the action of dilute acids, the tendons of the rat's tail behave differently from the tendons of the paws of the same animal and the tendons of other animals. More-

over, all the tendons of the rat, the rabbit and the dog, when treated with sodium hydrate and a dilute acid, yield Collagen B; but the tendons of bovines have been completely refractory up to the present time. Whether treated by an acid or by sodium hydrate, they swell and become transparent; if the action of the sodium hydrate is prolonged, they dissolve but they do not at any time yield substances coagulable by neutral salts.

We do not conclude from this that the different tendons are made of specifically different substances but merely that there are varieties in the chemical species *collagen*. While presenting many features in common, these varieties may be distinguished by properties just as striking as those on which many authors rely to make two distinct species of reticulin and collagen.

Summary

1. There exist different varieties of reticulin, all in continuity of substance with the collagen bundles in such manner that the connective tissue framework forms a single system. The elastic fibers, on the contrary, though mingled with the connective tissue framework, have no continuity with it.

2. The subcutaneous areolar tissue is well adapted to complete histological analysis. Thanks to the procedure of the edematous tumor and contrary to the general opinion, it can be demonstrated that the collagen bundles form a network like the reticulin. From the network of reticulin, which in this tissue is particularly delicate, to the network of collagen bundles, the transition is gradual and continuous, for the thickness of the fibers as well as for their coloring by silver.

3. When, in a tissue provided with a connective tissue framework, there are fixed cells other than fibroblasts, it is these cells that govern the arrangement of the reticulin. The fibroblasts, on the contrary, are scattered at random; they lodge where they can and they do not cause to be constructed around themselves any special arrangement.

4. In the adult, the fibroblasts people the connective tissue framework in almost its entire extent, but they are absent in some territories, such as the fat lobules and the hepatic lobules, where the framework is exclusively reticulated.

5. Treatment of the tissues with normal solution of sodium hydrate (4 per cent) does not hinder the impregnation of the reticulin in opaque black by silver.

6. Boiling the tissues, which causes progressive fragmentation of the reticulin, does not prevent the coloring of the fragments with silver as long as they persist. The reticulin of the pulp of the spleen does not resist boiling as long as that of the liver.

7. In silver impregnations, the color assumed by the fibers depends on physical conditions, on their thickness and conformation, but not on their chemical composition. This feature, then, cannot serve as a criterion to establish a systematic distinction between collagen and reticulin. The interfaces play an important rôle. Viewed with the ultramicroscope the collagen bundles present a broad, mirror-like surface, while the reticular fibers appear as sparkling lines.

8. Like reticulin, fibrin stains black with silver, on condition that the clot has been washed before fixation, but it does not stain if the albumins of the serum remain in contact with the fibrils.

9. Authors state that concentrated decoctions of viscera submitted to prolonged boiling do not jell on cooling. This result, the chief argument for the dualistic hypothesis, may depend on several causes; even if it does not rest on a parasitic phenomenon, in itself it is no more important than other variations observed in the attributes of the connective tissue proper, according to the tissue or the region involved.

10. Treated with a dilute acid, the tendons of the rat's tail, but not those of the paws, yield a substance (Collagen A) which coagulates in fibrils under the influence of neutral salts. These fibrils have all the properties of natural collagen except that they are more easily soluble in acids; they stain black with silver exactly like reticulin. The tendons of the rat and those of the rabbit and the dog, treated with sodium hydrate and then with a dilute acid, yield another substance(Collagen B) which also coagulates in fibrils with neutral salts. These fibers also stain black with silver. Tendons of bovines yield neither Collagen A nor Collagen B.

11. To say nothing of differences in the zoölogical series, homologous substances in the same animal present regional variations that selective stains do not reveal. Variations of this kind exist between the reticulins of various organs, but these differences are not of a

nature to establish a distinction between a collagenous system and a reticulin system; for nothing has been observed so far that warrants the supposition that the collagen part and the reticulin part of a given tissue differ in any way in the chemical constitution of their substance.

For the translation of this memoir, we have profited by the kindness and competence of Dr. George F. Laidlaw. We offer him our cordial thanks.

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DESCRIPTION OF PLATES

With the exception of Fig. 2, all the figures are photographs without retouching. All of the preparations were colored with ammoniacal silver.

PLATE 121

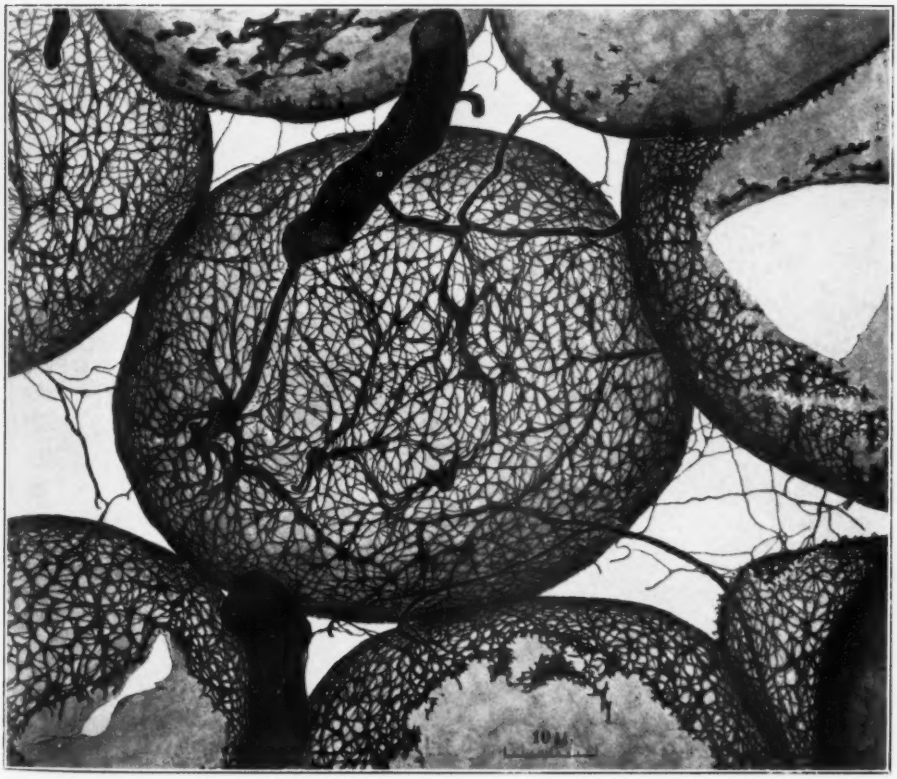
FIG 1. Artificial edematous tumor of the abdominal subcutaneous tissue of the rat. The reticulin is black. The collagen bundles in ribbon form are light yellow in the section, scarcely tinted in the photograph.

Fixation: bichromate-formol-uranium (Tupa's fluid). Celloidin section, 50 microns thick. × 250.

FIG. 2. Reticulin of the adipose tissue. Reticulated sheath of the fat cells. Some of the cells have been sliced by the knife and show their thin protoplasmic wall beneath the reticulum. Loose-meshed intercellular reticulum.

Fixation: formol-bromid. Frozen section, 100 microns thick. Drawn with camera lucida.

1



2

Nageotte and Guyon Reticulin

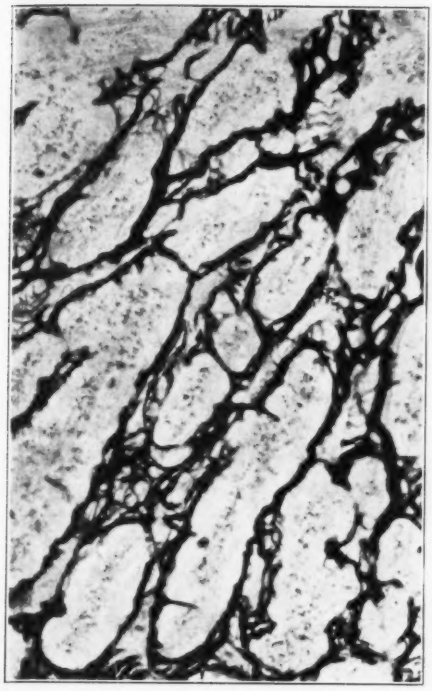
PLATE 122

FIG. 3. Reticulin of intact horse liver.
Fixation: Zenker's fluid. Paraffin section, 10 microns thick. × 500.

FIG. 4. Same liver after boiling one-quarter of an hour.

FIG. 5. Same liver after boiling half an hour.

FIG. 6. Dog's liver treated with 4 per cent NaOH for 24 hours. The hepatic cells have disappeared. The reticulum is intact morphologically and colored black.
Fixation: Helly's fluid. Paraffin section, 15 microns thick. × 500.



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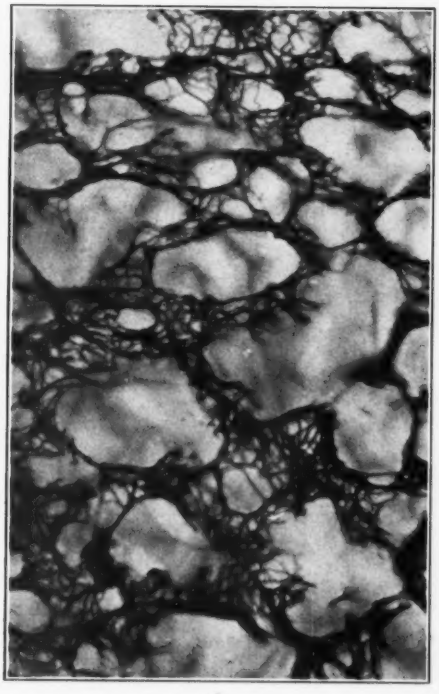
Nageotte and Guyon



6

Reticulin

PLATE 123

FIG. 7. Thin fibrinous clot of citrated rabbit plasma, formed on the slide by adding a solution of $CaCl_2$; washed; fixed in Helly's fluid. × 500.

FIG. 8. Capsule of horse liver. Reflection from the surface of the collagen bundles, colored yellow in the preparation. The light is regulated so that the interior of the bundles is not illuminated and remains absolutely black. Nothing is seen but the reflection from their surface.

Fixation: Zenker's fluid. Paraffin section, 10 microns thick. Ultramicroscope. × 400.

FIG. 9. Artificial clot of Collagen A (tendons of rat's tail), formed between slide and cover-glass by mixing the acetic acid solution with 1 per cent NaCl.

Fixation: neutral formol, 10 per cent. × 500.

FIG. 10. Artificial clot of Collagen B (tendons of rat's tail), treated first with 4 per cent NaOH, then by HCl, N:500. Same technique for the coagulation.

Fixation: Helly's fluid. × 500.

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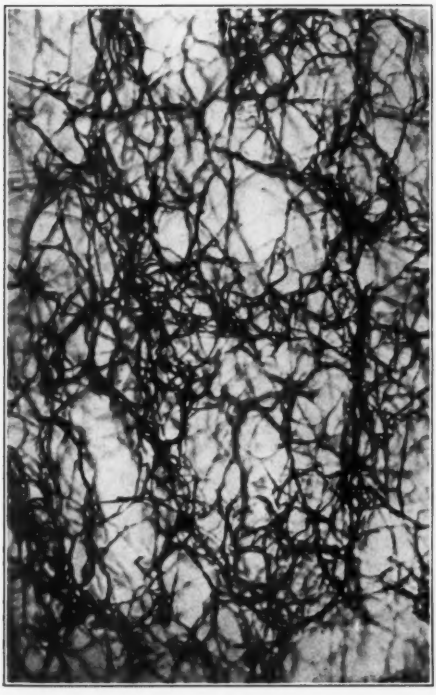
8



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Nageotte and Guyon

10

Reticulin

THE PATHOLOGY OF THE SPLEEN IN YELLOW FEVER*

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In a former communication (Klotz and Simpson, 1927) the pathology of the spleen in thirty-five cases of West African yellow fever was discussed. Since that time we have examined splenic tissues from many more cases, both human and animal, from America and Alrica, and these later studies have led to a more comprehensive consideration of the subject.

The pathological diagnosis of yellow fever rests mainly upon the recognition of the specific necrosis (Councilman lesion) in the liver; but not infrequently postmortem changes or poor fixation mask the true nature of the changes in that organ, and render the tissue unsuitable for study. In such cases considerable assistance may be obtained by the microscopic examination of the spleen, for the characteristic alterations in this organ are not so readily obscured after death. Again, it may occasionally happen that the particular block of tissue cut from the liver of a yellow fever victim will show no typical lesion. In such instances, observation of the changes in the spleen, and to some extent in the kidney, will prevent the true diagnosis from being passed over in the interim before further examination of the liver is possible. Aside from having a diagnostic importance, the pathology of the spleen is not without interest, for it illustrates a peculiar effect of the unknown toxin elaborated in yellow fever.

In the gross, the yellow fever spleen is characterized by the absence of distinctive changes. There is no appreciable alteration in size unless the individual has previously been infected with chronic malaria, which is frequent enough, or with some other complicating disease which produces splenomegaly. The organ is usually dark in color, flabby, and at times friable in consistency. On the cut surface the malpighian bodies are, as a rule, reduced in prominence and lacking in definition, but they may, on the other hand, occasionally ap-

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation.

Received for publication July 10, 1930.

pear quite prominent. Areas of degeneration or necrosis are never evident to the unaided eye.

The microscopic reaction is well marked. Different phases of it may be simulated and even duplicated in some other severe toxemias, but, on the whole, the reaction is sufficiently characteristic to distinguish yellow fever from other tropical infections with which, at times, it may be confused.

The appearance of the pulp is noteworthy, in so far as it presents two important findings. The first is the absence of an inflammatory reaction. In about 80 per cent of the cases there is a marked congestion of the sinusoids, which brings into unusual prominence the architectural structure of the pulp. Unlike the active splenic hyperemia of septicemia and typhoid, the congestion of the spleen in yellow fever is rarely accompanied by any increase in the white blood elements. One recognizes without much difficulty that the condition is essentially not of the nature of an acute splenitis. Secondly, the fixed tissues of the pulp are not hyperplastic. This is in marked contradistinction to the condition caused by relapsing fever, malaria, and Weil's disease, in which one observes a notable increase in the endothelial framework. As we shall see, proliferative changes in the yellow fever spleen are confined almost entirely to the malpighian corpuscles.

The reticulo-endothelial cells of the pulp frequently show primary enlargement and are somewhat more active than usual in phagocyting the red blood cells. In most cases the pulp contains large wandering cells of a peculiar type, which we shall presently consider.

The most striking changes are to be observed in the malpighian corpuscles. By examining the spleens of a large number of individuals who died at different stages of the disease, we were able to follow a definite sequence of alterations, the quality of which may be better understood if we first review briefly the normal cytology of the lymphoid follicle.

The observations of Thiel and Downey (1921) show that the malpighian corpuscle has a supporting framework, or reticulum, formed originally by slender strands of vascular endothelium which grow out from the central arteriole and its tiny branches. A lymphogenic function is conceded to the cells of this reticulum, but whether or not all the lymphocytes of the normal follicle are manufactured *in situ*, is in question. The splenic corpuscle often presents a germinal center

morphologically comparable to that of the lymph node. Under ordinary circumstances the endothelial framework is so distended with small lymphocytes that it is not recognizable, save in the periphery of the follicle where crowded concentric strands sharply divide the lymphoid elements from the adjoining pulp. The cells of the reticulum are connected only by slender cytoplasmic processes; their nuclei are slightly ovoid and tend to be somewhat vesicular.

Probably the earliest alteration found in the spleen in yellow fever is the appearance of large, unusual, mononuclear cells in and about the follicle. By the addition of these cells the follicle may become somewhat larger than normal. Usually its outline is blurred during this expansion process, but it may, on the contrary, become more sharply defined owing to the resistance of surrounding tissues.

The large mononuclear cells met with under these conditions are usually prominent, and often show a striking deviation from the normal. They are scattered through the lymphocytes, usually most numerous about the periphery of the corpuscle, where they sometimes form a mantle encircling the whole structure. In sparser fashion they are scattered over the pulp areas. They bear no regular relation to the fixed tissues and appear to migrate freely from one part of the spleen to another.

It is unusual to find any trace of germinal centers during this phase of the reaction. The large mononuclear cells dominate the whole follicle. These cells are somewhat variable in appearance but apparently belong to one type. They probably have their origin *in situ*. The structure of the nucleus varies from that which is typical of the majority, to that of large semidetached reticular cells, and it is not difficult to trace a transition between the two. Some of the intermediate forms are large, oddly shaped, and vesicular, but the majority are fairly uniform.

Turnbull (1913) described them thus: "The Malpighian bodies contain, in addition to lymphocytes, an increased number of cells which, in default of any generally accepted name, will be referred to here as 'free endothelial cells.' These cells are approximately round and occupy an area equal to from three to four-and-a-half red corpuscles. The protoplasm is non-granular and basophil. The nucleus occupies half or more of the cell. The chromatin is arranged as a, usually wide meshed, net of narrow rods, and is also massed as a stout capsule round each of the three of four large nucleoli.

The sharply defined nuclear membrane and the chromatin are deeply stained and contrast with the pale nucleoplasm."

The nucleus has a gently undulating surface which is characteristic; and not infrequently karyokinetic forms are manifested. Occasionally one or two of these cells may be found in a normal splenic follicle, especially in relation to the germinal center, while in many instances of acute febrile toxemias they are noticeably increased; but in the early splenic reaction to yellow fever they have a quantitative prominence which makes them a distinctive feature. This phase of the reaction is usually superseded, when death occurs, by changes in which the large mononuclear cell plays a less conspicuous part. Only about 20 per cent of our cases presented this early extensive mononucleosis.

Since the cell in question bears little or no resemblance to an endothelial leucocyte and is not phagocytic, the term "free endothelial cell" is probably misleading. It is more correctly designated as a primitive form, probably belonging to the lymphocytic series, and its presence is to be viewed as the result of a peculiar stress brought to bear upon lymphopoietic tissue. It is even more prominent in the lymph glands under identical circumstances. We shall, therefore, refer to this cell as a "primitive mononuclear," for the purpose of clearly differentiating it from the phagocytic large mononuclears of the blood and the cells of the reticulo-endothelial system.

Close upon the initial expansion of the follicle comes a diminution in the number of small lymphocytes. This is a constant feature of the yellow fever spleen. In many instances the primitive mononuclear cells are accentuated by want of the normal constituents of the follicle. The whole organ eventually becomes impoverished of small lymphocytes. The follicles become reduced in size and definition. Sometimes the disappearance of lymphocytes is so marked that many follicles virtually fade out of the picture, leaving only the arteriole and reticular skeleton.

As the small lymphocytes become less and less numerous, the primitive cells likewise gradually diminish, and the endothelial reticulum of the follicles becomes more and more hyperplastic. New cells bud off from the branching structure as from a vine. Not uncommonly one finds the lymphoid elements of the follicle almost completely replaced by a proliferation of small ovoid cells, which at

first sight appear to be degenerated lymphocytes, but on closer examination prove to be reticular cells, joined by frail cytoplasmic processes and growing in a compact mass. One can usually make out a few small lymphocytes and a few primitive mononuclear cells in the meshes of this endothelial hyperplasia.

With this activity on the part of the fixed tissues of the follicles, germinal centers spring into existence and frequently become prominent. These foci of growth often arise in relation to a small arterial twig, the wall of which appears to supply the parent tissue. Such germinal centers lack the loose cellular structure of the normal and are prone to degenerative changes.

When the endothelial hyperplasia reaches these proportions, degeneration invariably overtakes it. Indeed, at this stage the whole organ shows an intense toxemia, and has a peculiar appearance. Nearly all nuclei throughout the whole tissue tend to be somewhat washed-out or vesicular, and the cytoplasm is hyalinized and often vacuolated.

Many of the primitive mononuclear cells in pulp and follicle appear to be undergoing mitosis, but the chromatin material lacks the delicate structure of normal karyokinesis. With coincident evidence of retrograde changes in the cytoplasm, the mitotic process is to be viewed as a perverted response of a degenerating cell rather than as normal cell division.

Degenerative changes are most prominently featured in the malpighian corpuscles. The wall of the central arteriole is transformed into a pale homogeneous hyaline substance which spreads out as if it were in a plastic state. As a result the contour of the vessel becomes distorted. The tiny branches of the main arteriole are similarly affected.

The peculiar germinal center found in this phase of the reaction undergoes severe retrograde changes during the process of growth. It appears to be a fused mass of pale waxy cytoplasm, similar in consistency to the substance of the arteriolar wall. Often it contains nuclear débris and sometimes is the site of necrosis attracting polynuclear leucocytes. The mass is occasionally sharply limited by the surrounding tissues, as if it had developed by a rapid, expansive growth.

False germinal centers, as we term the abortive formations de-

scribed above, appear in the spleen in acute febrile states other than yellow fever, but they are rarely found in cases of chemical poisoning. They are to be interpreted as a response to a severe toxemia.

Primary enlargement of the newly developed reticular cells is observed around the periphery of the follicle. The nuclei are swollen and vesicular, presenting many bizarre forms which probably represent preliminary steps in the genesis of the primitive mononuclear cells.

About 20 per cent of our preparations show the presence of a few large multinucleated giant cells scattered through the pulp. They resemble the megakaryocytes of the bone marrow. Turnbull believed them to be derivatives of the large mononuclear cells. They are seldom quantitatively prominent, and they occur in the splenic reaction of other conditions. Indeed, it is not uncommon to find an occasional giant cell in the relatively normal spleen.

In those spleens which may be said to be in the end stages of the yellow fever reaction, one finds many curious masses of nuclear débris lying in the pulp. The fragments making up such a mass are usually in the shape of rings and crescents, and are jumbled together, forming a large, composite, irregular nuclear body without any apparent cytoplasm. The phenomenon may represent the phagocytosis of a number of small degenerate nuclei by a large pulp cell which has itself degenerated, or it may be simply a retrograde change in a multinucleate giant cell. We have not observed such masses in conditions other than yellow fever.

A moderate eosinophilia is often present in the spleen of advanced cases. The granulocytes are of a mature type and appear to be scattered through the pulp. There is no evidence of myelogenous proliferation within the spleen.

When the walls of the malpighian arteriole become severely damaged it is not uncommon to find hemorrhage into the follicle. Such a lesion is, however, of no moment.

Discussion

In a limited number of cases, we have had opportunity to compare the changes of the splenic corpuscles with those of lymph glands in the same individual, and we have found a striking similarity. These synchronous changes in the main lymphoid depots of the body lead to the conclusion that the changes in the splenic corpuscles are

but a part of the general reaction of the whole lymphopoietic system. The leucopenia associated with yellow fever is probably a related effect of the yellow fever toxin.

Toxins of different diseases frequently manifest some selective action on different parts of the hematopoietic system. Thus, many produce an anemia, others a polynuclear leucocytosis, and still others an abnormal activity on the part of reticulo-endothelial cells. None of these effects are observed in yellow fever, but instead there is a leucopenia and evidence of a progressive irritation and cytolysis in the lymphogenic tissues.

In the spleen there is an absence of the lesions which characterize the liver pathology of the disease. The Councilman necrosis is lacking, fatty change is negligible, and the nuclei present no suggestion of specific inclusion bodies.

Summary

In yellow fever, the spleen, like the liver, presents no distinctive gross features comparable to those seen under the microscope.

Active hyperemia is met with in about 80 per cent of cases, but is unaccompanied by a leucocytic infiltration.

There is absence of hyperplasia in the fixed tissues of the pulp.

Changes in the malpighian corpuscles characterize the splenic picture of the disease. Here, we recognize four phases of the reaction:

1. *Mononucleosis.* The type cell is an undifferentiated mononuclear derived from the reticular tissue of the follicle; it never entirely disappears during the entire course of the disease.

2. *Lymphopenia.* There is a striking loss of lymphocytes from the whole organ which persists throughout the reaction.

3. *Hyperplasia of the fixed tissues of the follicle.* False germinal centers are formed.

4. *Degeneration.* This is manifested throughout the whole spleen by vesicular nuclei and waxy degeneration of cytoplasm. False germinal centers undergo retrograde changes, amounting sometimes to actual necrosis. Pseudomitosis of the primitive mononuclears is observed. Large fragmented nuclear forms appear in the pulp.

A third of the cases show a few large multinucleate giant cells resembling megakaryocytes.

A moderate eosinophilia is commonly observed in the later stages of the reaction.

Changes in lymph glands parallel those of the splenic corpuscles, which is evidence of the fact that yellow fever toxin has a selective action upon lymphopoietic tissue.

We have found a careful examination of the spleen to be frequently helpful in facilitating a pathological diagnosis where yellow fever must be differentiated from other conditions giving rise to liver and kidney damage.

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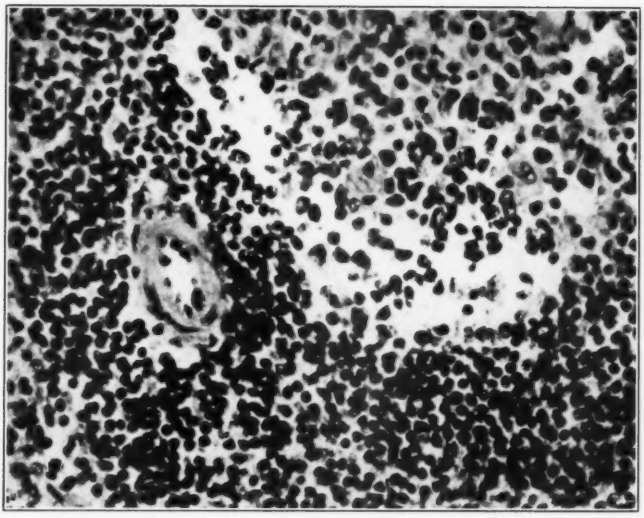
DESCRIPTION OF PLATES

PLATE 124

FIG. 1. Normal splenic corpuscle showing part of germinal center. From an individual who died instantaneously with a fractured spine. × 200.

FIG. 2. Early reaction in splenic corpuscle in yellow fever. A marked mononucleosis may be seen. × 200.



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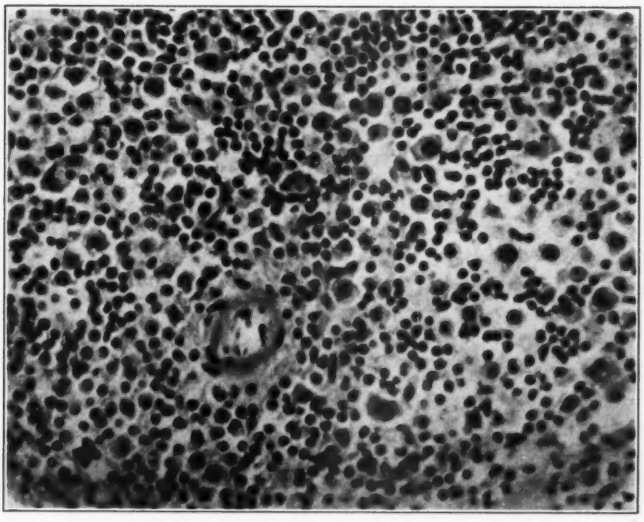

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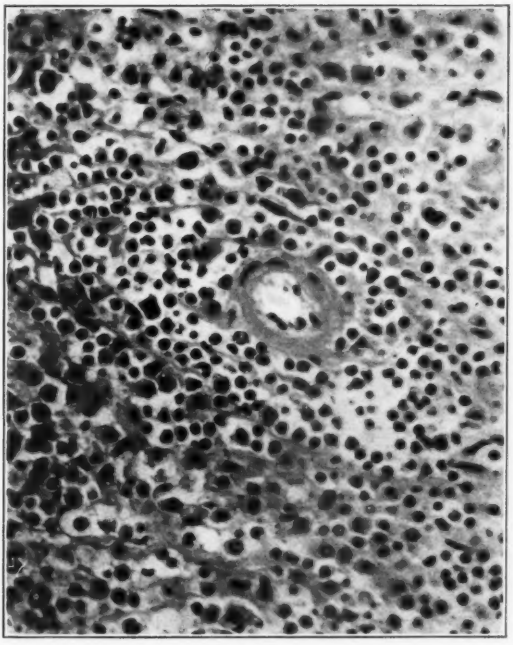
Klotz and Belt Pathology of Spleen in Yellow Fever

PLATE 125

FIG. 3. Second stage of yellow fever reaction in splenic corpuscle. Loss of lymphocytes is well marked. The reticulum of the corpuscle is prominent and large mononuclears may be seen budding from it. × 200.

FIG. 4. Later stage of yellow fever reaction in splenic corpuscle. The wall of the arteriole is swollen and hyalinized. The reticulum is hyperplastic and degenerate. On the left is part of degenerate false germinal center. There is almost a complete absence of lymphocytes. × 200.



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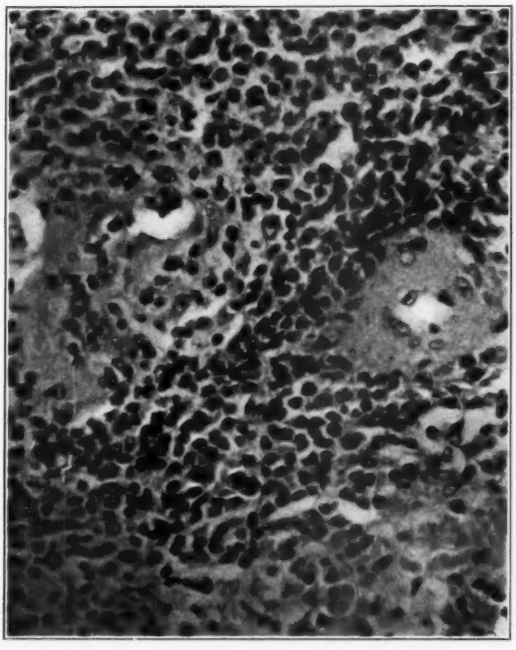

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Klotz and Belt Pathology of Spleen in Yellow Fever

THE PATHOLOGY OF THE LIVER IN YELLOW FEVER *

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Renewed interest has developed in the study of yellow fever since the reports of the West African Yellow Fever Commission of the Rockefeller Foundation, through whose efforts, in 1926, it was shown that the *Leptospira icteroides* is not related to the disease in West Africa, and that the guinea pig is not susceptible to the disease. Until 1927 yellow fever had never been experimentally produced in the lower animals. In that year the Commission undertook the task of finding a susceptible animal and was finally successful with the *Macacus rhesus*. Reproduction of the disease in this animal has facilitated research and has given pathologists an opportunity to learn something of the nature of the yellow fever virus. Although, up to the present, no visible organism has been discovered, it is known that the infecting agent possesses the qualities of a filtrable virus, both in its behavior under extraneous influences and in its attack upon susceptible tissues.

With the knowledge that yellow fever belongs to the group of virus diseases, we have given particular attention to the examination of the liver for the purpose of determining the nature of the specific changes which this organ undergoes during an attack of the disease. The importance of definite knowledge concerning cytoplasmic and nuclear changes is accentuated by the fact that not a few of the virus diseases show peculiar, and at times specific, intracellular changes by which they may be identified. The present study is confined to the liver, for the reason that no other organ or tissue has been found to show such constant characteristic lesions.

Our series of cases represents, without exception, natural fatalities from yellow fever. Human material was obtained from fifty West African cases and forty-three American cases. In addition, material from nineteen *Macacus rhesus*, made available through researches

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation.

Received for publication July 10, 1930.

carried out at the laboratories of the West African Yellow Fever Commission of the Rockefeller Foundation at Yaba, Nigeria, is included in the group. Care was taken to select only tissues obtained immediately after death and in which fixation had been properly carried out.

Several methods of fixation were used: Zenker, formalin, and Müller-formol. For general purposes of cytoplasmic and nuclear study the Zenker fluid without acetic acid served best. Formalin-fixed tissues of each case were cut on the freezing microtome and stained for fat with sudan-scharlach R and Nile-blue sulphate. Besides the routine hematoxylin-eosin stain, a number of other staining methods were applied to the Zenker paraffin sections, such as special Giemsa, phloxine-azure-B-bromide for intranuclear changes, Perle's stain for iron, Best's carmine for glycogen, Van Gieson's for stroma reticulum, and Goodpasture's fuchsin. We found it essential that the paraffin sections should not be more than four microns in thickness, otherwise the minute cytoplasmic and nuclear changes tend to be masked.

For comparison, forty-five cases of liver necrosis resulting from causes other than yellow fever were studied. This group included cases of acute yellow atrophy, eclampsia, Weil's disease, typhoid, relapsing fever, and six different kinds of poisoning.

In another paper we discuss the identity of the pathological lesions in the yellow fever cases occurring in Africa and in America. There is no longer need to designate the diseases upon these two continents by separate names, such as African yellow fever and American yellow fever. It is now an established fact that there is but one yellow fever, and the findings reported in this paper apply to the disease in both hemispheres.

Gross Pathology

Macroscopic examination of the liver of the yellow fever victim does not reveal constant and characteristic changes such as are seen under the microscope. It is not often that the amount of damage visible to the unaided eye arrests the attention in so striking a manner as does that which is shown by the microscope; and in no instance is it possible to make an accurate estimate of the extent of the liver necrosis by examination of the gross specimen alone. In this respect

the lesion encountered in yellow fever differs from that associated in our experience with acute yellow atrophy of the liver, and with toxic necrosis of the liver as seen in arsenical, carbon tetrachloride, and other hepatic poisonings. Moreover, since the liver lesion in yellow fever is more uniformly diffuse, except in rare instances in which the left lobe may be less involved than the right, the blotchy mottling observed in other conditions is absent. On the whole, the gross appearance of the liver, the organ most severely involved in yellow fever, is disappointing. It is necessary to make a careful microscopic study of the organ in order to recognize the characteristic lesions.

Since yellow fever is a disease largely confined to hot climates, postmortem changes in the body soon alter the *intra vitam* appearances. Many of the variations in the appearance of the liver which have been described were the result of postmortem processes. A difference of four to six hours in the length of time elapsing between death and autopsy will lead to quite marked variations in the color and external appearance of the liver. Furthermore, the boxwood color of this organ, which has so frequently been mentioned in the literature, makes its appearance only when the blood has been naturally drained away from the organ or has been pressed out after its removal.

The liver maintains its normal contour. It has a smooth and glistening surface, and there is no evidence of peritoneal change. Occasional small petechial hemorrhages may be observed under the capsule, but these are never numerous or large. In the fresh cadaver, not over three hours after death, the liver is of a reddish gray color shading into yellowish gray. At this time it is never yellow. However, if the autopsy is performed six to twelve hours after death, the liver is more decidedly yellow, but still has a reddish cast in its tissues. If the blood is allowed to drain from the liver after the organ is removed from the body, or if a portion of the liver is compressed between the fingers, the exsanguinated portions will appear quite yellow, sometimes even an ochre yellow, the color of boxwood. Only when postmortem changes have progressed, with their secondary tissue damage, have we found the liver of truly jaundiced appearance. At times the liver may appear quite red on its outer surface, with no evidence of extensive necrotic processes in the lobules. This is particularly true of fulminant cases, in which jaundice is not a promi-

nent feature. Jaundice of the liver and other structures of the body is most pronounced in individuals dying after the eighth or ninth day of illness. Ordinarily the size of the liver remains normal, or is slightly increased. The enlargement is never great and is caused by a slight diffuse edema involving both the stroma and parenchymatous cells.

On section there is evidence of cloudy swelling. The cut edges of the organ project upward and are everted. The larger portal vessels are sunk below the plane of the cut surface. The fresh tissue is a definite pinkish gray-yellow, a dull indefinite color, which becomes lighter and yellower as it remains exposed. The lobules are indistinct and cannot be clearly defined; frequently the central vein cannot be recognized. This lack of distinctness is no doubt due to the swelling of the lobules and the compression of many of the vascular channels, including, to some extent, the central vein. Some investigators have intimated that in the early stages of the disease the central vein area of the lobule is greatly congested, while in later stages a more uniform appearance of the cut surface is encountered. These differences in appearance can probably be accounted for by the differences in time after death at which the autopsy was performed. With the drainage of the blood from the liver, the structure of the organ becomes more uniform in appearance. It is true in the case of both humans and monkeys that when autopsies are performed immediately after death, the central vein is not uncommonly found to be prominent, as if in a state of passive congestion. This central congestion is, however, never so prominent or so persistent as the passive congestion caused by heart disease. We have never seen definite hemorrhagic necroses in the gross specimen of the liver.

The yellow color which makes its appearance diffusely in the liver and which may at times be described as clay color, at times as boxwood or ochre yellow, does not depend solely upon the presence of bile pigments, but also upon the fatty changes which always, to some extent, accompany this disease. Moreover, it is probable that the small quantity of bile which escapes during the acute disease is absorbed by the fatty materials and gives rise to the peculiar yellow color. As the quantity of fat present in the yellow fever liver varies tremendously, the associated changes likewise vary. The oft-repeated statement that the knife used in sectioning the liver appears greasy, and that quantities of fat may be scraped from the cut sur-

face, has been overstressed and exaggerated. Some livers are decidedly fatty, and in such cases a block of the tissue will float on water, but this is by no means a common finding.

Only the most careful examination with the magnifying glass will reveal evidence of necrosis. As the necrotic areas are not accompanied by interstitial hemorrhages, and as the fatty-icteric hue pervades the tissue diffusely, there are but few marks by which one may recognize the necrotic areas. Furthermore, this form of necrosis differs from that found in acute yellow atrophy, in that the necrotic liver cells do not undergo rapid dissolution; there is therefore relatively little actual loss of substance at the time of death, on the fifth or sixth day of illness. Hence, also, there is no evidence of the "pitting" or depressions which usually mark the areas of necrosis in the liver in other diseases. There are no thrombi in the portal system, and no change can be distinguished either in the hepatic artery or in the veins. The bile ducts also show no change, although the bile along their tracts and in the gall bladder may be quite viscid and thick.

Microscopic Pathology

Microscopic examination reveals a severity of liver injury which is in striking contrast to the small amount of damage visible in the gross. In the preliminary survey of the tissue a marked disorganization of the parenchyma is at once apparent. There is jumbling of the liver cords to a greater or lesser degree, with proportionate distortion of the sinusoids. The injury consists in a process of necrosis and necrobiosis which is remarkable in several respects; the entire lesion is of a non-inflammatory character and lacks an exudative response save in the presence of a secondary process; it is more diffuse than focal in character, but in the majority of cases involves chiefly the midzones, tending to spare the peripheral and central parts of the lobules; evidence of a rapid autolysis is lacking so that there is no alteration in contour nor reduction in size of the lobules, such as one might expect in consideration of a widespread cell destruction; the structures of the portal sheaths are practically unaffected, and interstitial hemorrhage is rarely found.

A peculiar form of necrosis invariably dominates the microscopic picture, but fatty change and cloudy swelling contribute to the lesion in every case. These three degenerative processes, which we

shall presently consider in turn, are to be viewed as independent effects of the blood-borne damaging agent of the disease.

There is a wide variation in the intensity of liver injury, but in every instance well marked and characteristic changes are found. The severity of the lesion bears no fixed relationship to the clinical severity of the attack. Some very acute and rapidly fatal cases show only a minimal liver lesion.

Cytoplasmic Changes

Fatty Deposits: The occurrence of fat in the liver cells, though somewhat variable in amount and distribution, is constantly observed and must be considered as characteristic of the disease. It is laid down in large and small droplets: the former occupy the better preserved cells, the latter the more degenerate cells. Large droplets were met with in only about one-half of our cases, but the finely divided or granular fat was always present. Both forms stain alike and probably represent expressions of the same process, whether it be an accumulation of physiological fat due to a local failure of fat metabolism, as Mallory believes, or a lipoid deposition in the cytoplasm resulting from disintegration of compound bodies. It is not possible to distinguish between fatty infiltration and fatty degeneration in yellow fever livers. The most one can say is that the larger fat droplets are found in the necrobiotic and partly injured cells, which, in the matter of functional activity, stand midway between the apparently healthy peripheral cells and the necrotic cells. Necrotic cells contain only the finely divided fat which may appear in considerable quantity.

The total quantity of demonstrable fat in the liver lobules varies from case to case, and we have never been able to correlate the extent of the fatty change with any particular character of the disease. At times, in fulminant cases, heavy deposits of fat appear in the liver, but at other times a similar condition is discovered in less intense cases where death was caused by renal complications. It must be remembered that extremely fatty livers in which the tissue is loaded with a fat deposit sufficient to catch the eye at autopsy and lead to the comment of "greasy liver," are unusual. When such a liver is found, the fat appears in large globules and occupies the cells in all zones of the lobule.

The zonal distribution of fat is often considerable, and shows itself chiefly in relation to the midportion of the lobule. This zone, even when showing a well marked zonal necrosis, contains a sufficient number of necrobiotic cells to make the deposit prominent. However, in other cases where the midzone necrosis is well advanced, a fatty zone may delimit the necrotic area on each side so that an apparent peripheral as well as central zone of fatty degeneration is found. Moreover, the fat may appear in unequal quantities and distribution in the different lobules of the liver.

When stained with Nile-blue sulphate, some of the granules in the necrotic cells manifest the reaction of fatty acids or their compounds, but throughout the lobule the droplets are almost entirely of the nature of neutral fat.

Cloudy Swelling: Some degree of cloudy swelling is constantly present in the liver parenchyma. We have found scarcely a single instance of a normal liver cell in all our preparations. This type of degeneration is manifested chiefly by a finely granular appearance of the cytoplasm and a moderate edema in the better preserved cells. It is a diffuse and even change of the cytoplasm involving little alteration in staining qualities. The granules are only obscured when the cytoplasm undergoes coagulative hyalinization. When the necrotic process occupies chiefly the midzone of the lobule, the cells in both the peripheral and central zones show cloudy swelling. The swollen appearance of many of the cells is, of course, accentuated by their engorgement with fat. It is probably owing to this edema and fat deposit that the liver cells are larger than normal, accounting for the slightly increased bulk of the liver despite the presence of parenchymatous necrosis.

Hyaline Necrosis (Councilman Lesion): The degenerative process involving the liver lobule may develop into an advanced stage of necrobiosis with the manifestation only of fatty change and cloudy swelling. The cytoplasm of the cell under such circumstances is markedly rarefied and granular, while the nucleus is swollen and hydropic. But it has not yet passed the point where recovery is impossible. It is only with the manifestation of hyaline necrosis, referred to elsewhere as the Councilman lesion, that the cells disintegrate and death occurs. This peculiar change, more than any other, characterizes the liver lesion in yellow fever. In our experience it is present in all cases of natural fatality from the disease, whether hu-

man or animal. The necrosis may apparently supervene at any stage of the degenerative process, though it is probably preceded in all cases by some degree of fatty and cloudy change.

The proportion of the lobule actually necrosed varied roughly from 5 to 100 per cent in the different individuals of our series, with the mean average somewhere about 80 per cent (see Chart I). An appreciable number of the human cases showed comparatively slight damage to the liver, having less than a tenth of the hepatic cells necrotic. It is in cases of slight damage, such as these, that the

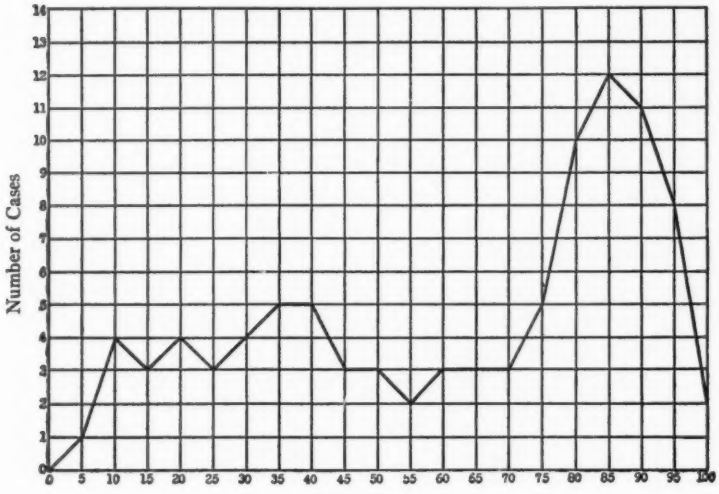

CHART I

Percentage of Average Lobule Destroyed by Necrosis

peculiar distribution of the necrosis is best demonstrated. It runs to all parts of the lobule and is variously described as diffusely sprinkled, scattered, sporadic, or patchy. The cells of a given liver column are affected discontinuously; single cells at irregular intervals are attacked in part or as a whole, or small groups of two or three adjacent cells fall prey together.

As greater proportions of liver cells become necrosed, midzonal destruction is more and more emphasized. This localization, stressed by Rocha Lima, is well marked in livers which have between 30 and 90 per cent of the average lobule involved in necrosis. Midzonal

areas of concentrated destruction are, however, in the majority of cases, ill-defined and shade off peripherally and centrally into the sporadic lesion which reaches to the outer and inner limits of the lobule. A narrow zone of cells surrounding the portal sheath is the last part of the lobule to be destroyed; its preservation is another of the characteristic features of the yellow fever liver.

In most cases both the sporadic and zonal distribution of necrosis may be identified, but the former is constant and specific, while the latter is uncertain as a diagnostic criterion. The smaller the proportion of necrosed cells in a lobule, the more scattered or sporadic is the lesion, and the less definite the midzonal concentration. Conversely, when the midzonal emphasis is lost because of complete destruction of the lobule, the sporadic selectivity may still be distinguished in the varying intensity of necrosis from cell to cell.

Not all parts of the liver are affected to a like degree. It is usual to find the lobules in any one microscopic section more or less uniformly involved, but in a few instances exceptions to this rule are encountered. Lobules lying side by side may be affected in a very unequal fashion. In two cases blocks of tissue cut from the left lobe of the liver failed to show lesions characteristic of yellow fever, while blocks from the right lobe, subsequently examined, gave a typical hepatic picture of the disease.

Necrosis of the parenchymatous cells, whether scattered sporadically or arranged more or less in zones, is always readily recognized by the peculiar, acidophilic, hyaline change of the cytoplasm, which has no counterpart in other diseases. Scattered focal necroses have been described in a number of diseases, and midzonal necroses may arise in severe sepsis and peritonitis, but in none of these do we find the characteristics of cell necrosis as we find it in yellow fever.

Ordinarily the parenchymal cytoplasm is neutrophilic or faintly basophilic, according to the degree of differentiation. The necrosed cells and parts of cells have, by comparison, a marked affinity for eosin and like dyes, which makes for sharp contrast with the surrounding cells (Fig. 1).

In consistency the necrosed parts are denser and more refractile than normal cytoplasm. Their substance is of a homogeneous character, which, coupled with acidophilic properties, has given rise to the designation "hyaline necrosis" or "hyaline bodies." They are, as a rule, honeycombed with small vacuoles, which in frozen sections

are seen to contain granular fat. Very often remnants of nuclei and granules of yellowish bile pigment are seen within the hyaline mass.

The cells or parts of cells undergoing necrosis invariably become rounded into spherical forms. This distinctive morphological feature adds to the prominence of the hyaline bodies. These bodies tend to shrink in the process of preparation so that they are often emphasized in outline by a narrow clear zone, artificially produced. In many specimens such discrete bodies are quite numerous and at one time they were thought to be amebae or some other unicellular parasite — an interpretation which their morphology does, indeed, suggest, although their variation in size discounts it. They range roughly from five to twenty-five microns in diameter.

Frequently an elongated nucleus is observed, flattened against the outline of the necrosing cell in such a way as to simulate the contour of a signet ring. Rocha Lima interprets these nuclei as belonging to wandering cells which have penetrated the necrotic mass of cytoplasm; but whatever their origin, signet ring forms are often quantitatively and morphologically prominent in the parenchyma of the yellow fever liver.

The spherical hyaline bodies soon lose their contours and disintegrate. This process is hastened by postmortem change, poor fixation of the tissue, and advanced stages of necrosis. Thus, in many instances, the identification of the Councilman change rests more upon the staining reaction and the consistency of the affected cells and cell parts than upon their morphology. In cases where the liver damage is maximal, whole lobules may be virtually littered with amorphous fragments of necrotic cytoplasm, which, however, retain their distinctive hyaline character.

The technical process of preparing microscopic sections probably exaggerates the natural tendency to crumble which the hyaline masses possess. Portions of necrosed cells often appear to lie free in the sinusoids. This phenomenon led Councilman to speculate as to whether emboli might not thus arise. It seems probable, however, that such fragments are detached by the microtome knife, and are not free within the blood channels during life.

In many instances fragments of hyalinized cytoplasm closely resemble distorted red blood cells, and on casual observation, the two might easily be confused. Not a few authors have insisted upon the frequency of interstitial hemorrhage in the yellow fever liver, but we

believe that this erroneous interpretation has arisen through failure to make a critical distinction between erythrocytes and necrotic fragments of cytoplasm. The former are never vacuolated, as are the latter.

The nature of the distinctive hyaline change is not clear, except that it represents a coagulative, rather than an autolytic process. There occurs a condensation of the cytoplasmic substance in which may remain incorporated some of the fat and pigment contained in the cell before its necrosis. The development of the discrete hyaline spherules within the membrane of the living cell probably represents a localized coagulative change, wherein a diffusely hyalinized patch of cytoplasm becomes isolated and assumes a globular shape.

In only one of the forty-five cases of liver necrosis in affections other than yellow fever, which were studied for purposes of comparison, did we find a condition resembling the peculiar pathology of yellow fever. This was a case of cellulitis and pneumonia in an infant, presenting scattered necrotic cells in the liver parenchyma. These cells were completely hyalinized and did not show the partial hyaline coagulation of cytoplasm so often observed in yellow fever. The necrosis had changed the staining properties of the affected cells from neutrophilic to acidophilic, but failed to give rise to that homogeneous change which serves to accentuate the hyaline masses in yellow fever. Fine fatty vacuoles were also absent from the hyalinized cytoplasm, which constituted a further difference; and the distribution of the necrotic cells was less diffuse and more focal in nature than in yellow fever.

Nuclear Changes

In the Human Liver: Nuclei of the liver cells in yellow fever react in a variety of ways, many of which are not distinctive of the disease. The commonest change is a combination of nuclear edema, chromatolysis, and acidophilia of the intranuclear constituents, and although this combination is found in nearly all yellow fever livers, it also occurs frequently in other conditions.

These changes are confined almost entirely to the cells of more normal appearance which are affected only with cloudy swelling and fatty vacuolation. The intranuclear appearance differs considerably in frozen and in paraffin sections. A comparison of the two shows

that in the latter there are many changes which may well be attributed to the technique of dehydration. In the former, the nuclear membrane is well filled with a watery nucleoplasm in which small, dull, chromatin granules are scattered more or less evenly. The nucleoli can easily be identified but they are not prominent. In paraffin sections, on the other hand, the same nuclei usually present a "washed-out" appearance. Much of the chromatin appears to have been lost, while the remainder is granular and collected in small irregular clusters on the inner surface of the nuclear membrane and the outer surface of the nucleolus. The remaining intranuclear space is optically clear. The chromatin shows a loss of affinity for the basic dyes and varies in color from bluish purple to dull red. In this type of nuclear alteration there is no apparent cleavage between the basophilic and acidophilic elements of the chromatin, but the whole undergoes a more or less uniform change. This is the important point of distinction between the non-specific nuclear change, and the so-called inclusion phenomenon in which a definite separation of blue-staining and red-staining chromatin appears to take place. As a rule, the nucleoli stand out very prominently; occasionally they take on a faint blue tint, but for the most part they are stained reddish pink. There is a striking frequency of two and three nucleoli within a single nucleus. Where multiple nucleoli are not seen, there is often marked increase in the size of the single nucleolus.

A second type of nuclear change is much less prominent, but is more typical of yellow fever in a qualitative way. We refer to the so-called "inclusion bodies" and alterations associated with them. The term "nuclear inclusion bodies" has come to have a definite meaning which one is at a loss to convey tersely except by some such arbitrary designation. It is misleading in the sense that it implies the presence of extraneous matter within the nucleus. This conception is by no means justified; the term is applied to bodies whose nature and mode of development are comparatively unknown; therefore, it does not lend itself to accurate understanding unless used by the author in a specific and restricted sense.

The nuclear inclusions first described by Lipshütz as peculiar to lesions produced by herpes virus are of a definite character, both quantitatively and qualitatively. They occur abundantly in experimental lesions and are easily demonstrated by the ordinary hematoxylin-eosin method. For these reasons herpetic inclusions have

been studied more extensively than other types, and have come to be looked upon as the standard example. From a study of herpetic inclusions in all stages of their development, supplemented by a review of the literature, the following broad definition suggests itself as a criterion by which we may judge the presence or absence of the phenomenon in yellow fever livers: An intranuclear inclusion body is (1) not a preformed part of the nucleus, as for instance, chromatin or nucleolus; (2) markedly acidophilic, so that it contrasts with other parts of the nucleus when stained by ordinary methods; (3) associated with preservation of the nuclear membrane and migration of basophilic chromatin to the periphery of the nucleus; (4) surrounded or partially surrounded by a clear space; (5) not possessed of a constant size or morphology; (6) single or multiple in a single nucleus; (7) made up of fine granules, which are more or less uniform in size; (8) formed of an unknown substance which has been shown by Cowdry (1928) to possess certain constant microchemical properties; (9) not of the nature of an artefact since Goodpasture and Teague (1923), and Cowdry and Kitchen (1929, 1930) have demonstrated it in the fresh unstained cell.

Cowdry and Kitchen (1930) have completed a much more exhaustive study of nuclear inclusions in human yellow fever than opportunity has permitted us to undertake. These authors have contributed a comparative description of the known types of specific nuclear bodies as they occur in fresh, frozen, and fixed tissues, including microchemical properties and staining reactions to various dyes. The authors direct special attention to the peculiarities of the inclusion phenomenon as it occurs in yellow fever; they were able to demonstrate specific nuclear bodies in ten out of thirty-nine human cases.

We observed definite nuclear inclusion bodies in twenty-three of ninety-three human cases. In each of these the typical bodies described by Cowdry and Kitchen were found, often in such numbers as to form a striking feature of the histological picture. Twenty-seven additional cases presented a nuclear change which we interpreted as being closely related to the formation of inclusions. Indeed, they conformed to the picture which Cowdry and Kitchen describe as the end-stage. This atypical form was seen also in the twenty-three cases positive for typical inclusions.

The nuclei containing the inclusions are, as a rule, quite small and,

in further contrast to the swollen nuclei previously described, are confined almost entirely to necrobiotic or actually necrotic cells. The nuclear membrane stands out sharply as if outlined in India ink. It is invariably finely beaded with small dark droplets and granules of chromatin. Not infrequently the nuclear outline is distorted in shape. The end-stage inclusion fills the whole intranuclear space with a dull, pink, homogeneous ground substance. Such an inclusion has lost its distinctive character, and cannot be accurately spoken of as a specific change, for several of the control livers present similar forms.

Of the whole series, then, twenty-three were positive, twenty-seven doubtful, and forty-three negative for intranuclear inclusion bodies.

In view of the comparatively small proportion of cases manifesting the phenomenon, we investigated the series carefully to ascertain whether any factor could be correlated with the presence or absence of inclusions. We found that age, sex, and race have apparently no bearing on the matter. The length of time intervening between death and autopsy was equally variable, and within the usual limits in both positive and negative cases. Cases with Zenker-fixed tissue show a slightly higher incidence of inclusion bodies than those with formalin-fixed material. However, the method of preservation seems to be less important than the length of time the tissues have been kept. The incidence of inclusions was four times as high in the African as in the American cases of our series, but this was probably due in part to the prolonged formalin fixation to which the bulk of the American tissues had been subjected. From the examination of different groups of cases which had occurred in different epidemics, it was suggested that some strains of virus might perhaps exceed others in their ability to produce specific nuclear changes; but if one factor stands out more than another in relation to the presence of inclusion bodies, it is the duration of the illness. Among the positive cases, none had been ill longer than 6 days, and the average duration was 3.8 days. The negative cases ranged between 3 and 12 days of illness, averaging 5.8 days. These observations bear out Torres' suggestion that inclusions are present only during the period when the virus is free in the blood stream. There remains to be explained, however, the absence of inclusions in certain of our cases that died in the early (infective) period of yellow fever, and the conclusion pre-

sents itself that the demonstration of the inclusion phenomenon is dependent upon a multiplicity of factors, probably in the following order of importance: duration of illness, extent of postmortem changes, method of fixation of tissues, length of time of preservation, staining technique, and peculiarities of the virus involved.

In the control group, with one exception, we found no inclusion bodies. The exception occurred in the liver of a premature infant that had died from undetermined causes 14 days after birth. We are indebted to Dr. S. B. Wolbach of the Harvard Medical School for the tissue. The inclusions were very clear-cut and numerous, but they resembled the inclusions of herpes more than the yellow fever variety, and the associated liver damage was quite unlike that of yellow fever.

Nuclei of the parenchymal cells do not manifest pyknosis when affected by the Councilman lesion; they either pass through the stages of inclusion-body formation, or undergo an allied process whereby basophilic properties are completely lost. Such a nucleus, when incorporated within a hyaline body, shows as a dense acidophilic mass. In other conditions, where the liver cells suffer damage, pyknosis of the affected nuclei is common.

In several of our specimens we noted a nuclear change which resembled mitosis. It was confined to cells which, as far as we were able to judge, had retained their vitality even though the cytoplasm was usually granular and heavily vacuolated. The nuclear membrane was absent and the chromatin arranged in a cluster of fairly regular rod-shaped masses or granules which took a dark brownish purple stain. At times these fragments were scattered through the cytoplasm as if by an explosive process and in such instances they looked not unlike parasitic forms. At other times two irregular groups were formed so that the whole resembled a diaster phase of mitosis. Unquestionably this is the change which other investigators have interpreted as a sign of cell division, but we do not believe that it can properly be viewed as such. If there was active cell proliferation during the acute and fatal stage of the disease, as some authors would lead us to believe, we should expect to find all stages of karyokinesis, but in all our specimens we have never observed an actual transition from prophase to telophase. An occasional true mitotic figure is seen, having polar bodies and spindle threads, but the great majority of these peculiar nuclear forms show no such evidence of cell division.

The associated retrograde changes in cytoplasm also argue against normal mitosis. All the evidence, on the contrary, points to a degenerative change in the nucleus in which the metaphase and less often the anaphase of mitosis is simulated. We are of the opinion that active liver regeneration is at a low ebb during the acute stages of the disease and that few true mitoses are found in any part of the lobule. In reviewing the cases it was surprising to note that karyokinetic figures were no more frequent in individuals surviving the tenth to twelfth day of illness, in whom liver necroses were still the outstanding lesion, than in those who had died as early as the third day.

In the Macacus Rhesus Liver: The nuclear inclusion phenomenon as it relates to yellow fever, was first observed in the livers of experimental animals and described by Torres in 1928. Subsequently, as we have seen, the phenomenon was identified in the human liver. The incidence and character of intranuclear inclusions in the monkey are, however, quite different from those in man. Cowdry and Kitchen (1930) have dwelt fully upon this interesting feature and have advanced some plausible suggestions as to the cause of the discrepancy, but an adequate explanation is lacking.

Of our total nineteen *M. rhesus* livers, seventeen were positive for typical inclusion bodies, one was doubtful, and one negative. The nuclei containing specific bodies were relatively larger than the corresponding nuclei of the human liver. Moreover, in the experimental animal the inclusions were associated with fatty and granular changes in the cytoplasm and appeared to precede or be quite independent of the Councilman lesion. In the human cases, on the other hand, when inclusions did occur, they were directly related to necrosis. In other minor respects the nuclear changes of the two species were similar.

Other Changes

About 80 per cent of the cases, human and animal, showed a moderate deposit of finely divided granular pigment in the parenchymal and stellate cells. It was a dull yellowish brown in color. Often the pigment lay in a fairly well defined lacuna bordering on the nucleus; at other times it was scattered freely throughout the cytoplasm. The exact nature of the pigment was obscure but it was probably of biliary origin. Attempts at differentiating hemofuscin and lipofuscin

from bile pigment were unsatisfactory and, in our opinion, of no value. Sometimes thin worm-like threads of inspissated bile could be seen within the canaliculi. The large, homogeneous globules of bright yellow bile which are often quite prominent in the livers of acute yellow atrophy cases, are not found in yellow fever.

Minute quantities of iron, located chiefly in the Kupffer cells, but present also within the liver cells and in the interstices of the portal stroma, could be demonstrated occasionally by means of Perle's stain. This pigment is to be interpreted as altered hemoglobin, and its presence is probably not related to the attack of yellow fever.

Of considerable importance is the apparent loss of glycogen from the liver as demonstrated by Best's carmine stain. Owing to the difficulty of obtaining fresh human tissues properly fixed in alcohol, it was possible to carry out the test satisfactorily on *M. rhesus* material only. Glycogen is found to disappear from the liver cells soon after the onset of the severe symptoms and when the liver is showing its early specific hyaline necrosis. Soon the liver is all but entirely depleted of its glycogen deposit. In general, the diminution of glycogen storage is proportionate to the intensity of the liver injury.

In a former communication (Klotz and Simpson) damage to the Kupffer cells of the liver in yellow fever has been dealt with at length. It will suffice, therefore, to review the important changes briefly. Hyperplasia of the stellate cells is found in the late stages of the less fulminant cases and appears to be associated with clinical jaundice. These cells are increased not only in number, but in size as well. In cases where parenchymal damage is more severe, the Kupffer cells are often swollen and granular; their nuclei may be pyknotic or otherwise degenerate. The cytoplasm frequently shows bile and iron-containing blood pigment, as well as fine hyaline droplets and, sometimes, granular fat. Occasionally these cells also show the presence of black iron-free pigment of malarial origin. The cells are commonly seen to lie loosely in the blood sinusoids, while the endothelial cells lining the sinusoids are fat and small and lie closely attached to the liver cords. In many sections the nuclei of the Kupffer cells are unusually prominent and much better preserved than those of the parenchymal elements.

In cases of severe necrosis the sinusoids may be disrupted in some part of their course, but usually they may be traced winding tortuously between jumbled cords. They are often compressed by swollen,

fatty cells, but from this, as Seidelin has said, the conclusion is not warranted that obstruction has existed *in vivo*. In twenty-four cases, animal and human, a considerable amount of fresh blood congested the sinusoids in the midzonal regions where necrosis was most severe, but the presence of actual hemorrhage into the cord interstices was very unusual. Van Gieson's stain, employed in instances of severe parenchymal necrosis, demonstrated remarkable preservation of the fine stroma reticulum which is probably an additional factor in preventing hemorrhage from the sinusoids even after the liver cords have disintegrated. The majority of our cases presented a bloodless condition of the liver and there was a total absence of fibrin or thrombi in the blood channels.

One of the outstanding features of yellow fever is the lack of visible damage in the stroma. Herein lies one of the great differences between yellow fever and certain other liver necroses. Undoubtedly the preservation of the stroma accounts in part for the fact that there is no collapse of the lobules, no matter how intensive the damage to the epithelium may be. Occasionally the intralobular ramifications of Glisson's capsule are slightly swollen, but there is no fibrous response. In the portal areas of the African cases one sometimes encounters a moderate degree of cirrhosis which is obviously quite independent of yellow fever.

Another striking attribute of yellow fever lesions in the liver is the lack of inflammatory reaction. Scattered leucocytes are found in some liver specimens, both intra- and extravascular, more frequently in the monkey than in man, but frank infiltrations are not found in the parenchyma. Limited numbers of polynuclear and endothelial cells may appear where cellular disintegration has taken place, but unruptured necrotic cells do not appear to stimulate leucocyte migration. The point we wish to emphasize is that the liver lesion is distinctly not of the nature of a hepatitis.

The bile canaliculi in the liver can frequently be identified. They are invariably in a contracted condition and contain only minute amounts of dull, finely granular bile pigment. The bile-ducts of the portal sheaths show no damage to their epithelium beyond an occasional instance of fatty change. In a larger proportion of our human cases, slight to well marked lymphocytic infiltration was observed in the portal sheath in close relation to these biliary channels. Sometimes degenerated forms of endothelial cells were present instead of

lymphocytes, and occasionally both shared in the reaction. The high incidence of this lesion is striking, but its significance is obscure. We have observed the same lesion less frequently in the other forms of liver necroses. In the West African adult, irrespective of yellow fever, it is rare to find a liver that is normal in the portal zones.

Discussion

The liver lesion in the yellow fever of Africa and of America is the same and consists of a non-inflammatory degenerative process involving the parenchymal cells alone. The Councilman lesion, in our experience, is the most characteristic feature of the histological picture, and forms the best basis for a pathological diagnosis of yellow fever in the human subject. It is constantly observed and cannot be confused with other types of liver necrosis described by Mallory, Opie, McCrae and Klotz, and others.

In the early stages of necrosis the hyaline bodies of the yellow fever liver resemble the cytoplasmic inclusions of other virus diseases. In the color plate there are represented intracellular hyaline globular masses which look not unlike Negri bodies, except for the absence of basophilic granules. Not only in consistency, staining qualities and morphology does the similarity suggest itself, but the distribution in cells and the absence of inflammatory change in relation to them are characteristic of the intracellular changes in virus diseases. It is not our intention to suggest that Councilman bodies are inclusions in the same sense as those of Guarnieri, Bollinger, and Negri, but a certain analogy does exist. Later stages of the necrosis show a massive involvement of liver cells in which discrete hyaline bodies are no longer apparent and the analogy to the inclusion formation disappears.

The peculiar distribution of the Councilman lesion cannot be explained. Zonal necrosis cannot, as Chiari suggests, be accounted for by the peculiar vascular supply of the liver, for Whipple and Sperry (1909) showed that ligation of the hepatic artery and synchronous establishment of an Eck's fistula did not alter the characteristic zonal distribution of chloroform necrosis. In the yellow fever liver it is evident that we are dealing with the effect of a blood-borne toxic agent, but like the character of the agent itself, the peculiar selective localization of the necrosis is at present beyond accurate explanation.

Special attention is directed to the non-inflammatory character of the entire liver lesion, and to the lack of autolysis in association with the necrosis. We believe that these features favor an early, complete, and scarless regeneration of the liver tissue in cases which survive the disease, and we propose to consider this aspect more fully in another article dealing with regeneration.

The identification of nuclear inclusion bodies in relation to yellow fever brings to light an unique cellular change, which so far as we know, is peculiar to virus diseases. In this respect the findings brought forward by Torres, and later by Cowdry and Kitchen, constitute an important contribution to our knowledge of the nature of the disease. It must be remembered, however, that nuclear inclusion bodies are found only in a small minority of human yellow fever cases, and that they cannot, therefore, be considered of diagnostic importance. Moreover, the positive identification of specific nuclear inclusions in yellow fever tissues is not an easy matter. Indeed, non-specific intranuclear bodies may often simulate and be confused with true inclusions so that it is not desirable in any case to have the diagnosis rest upon this distinction. Some idea of the uncertainty attending the identification of these inclusions may be gleaned from the fact that in their first publication (1929) Cowdry and Kitchen claim twenty-two out of twenty-five cases positive for inclusions, while in their later treatise (1930) they are able to list only eight of the same series as positive. If the complete microscopic picture be taken into account, there will be found ample evidence, in the human cases at least, upon which to make the diagnosis, without reference to nuclear changes.

The presence of intranuclear inclusion bodies is a diagnostic criterion of greater importance in *M. rhesus*. The Councilman lesion was present in all of our nineteen cases, but was less prominent than in the human; it is not accompanied by the same contrast in staining (acidophilic) properties, and seldom gives rise to discrete hyaline bodies. On this account it is sometimes difficult to recognize, but when found it may be taken as pathognomonic of yellow fever. It is noteworthy that cytoplasmic changes in the *M. rhesus* are less characteristic than in man, while the reverse is true of nuclear changes.

Because of failure to distinguish between Weil's disease and yellow fever, the literature has become burdened with a confusion of contradictory observations from which it is impossible to draw an ac-

curate idea as to the histopathology of either disease. Many noteworthy investigators (Noguchi, Müller) have described what they thought were yellow fever lesions, when in reality the disease they were dealing with was infectious jaundice, or an allied process.

It is regrettable that the contributions of Councilman (1890) did not receive a wider circulation in the literature. To him we owe not only the first, but the best and most lucid description of the peculiar liver necrosis which is now associated with his name. He did not, however, recognize the lesion as specific for yellow fever. Since Councilman's description other authors, including Rocha Lima (1912), Turnbull (1913), Chiari (1925), Torres (1926), Hudson (1928), Couto and Rocha Lima (1929), and Hoffmann (1929) have commented upon the hyaline cytoplasmic change, but with the exception of Penna and Figueiredo (1929) no one has sufficiently stressed the differential importance of it.

The distribution of the necrosis rather than its minute character has been the more vital issue among investigators, and many excellent illustrations of zonal destruction appear in the literature. Councilman in 1890 noted that the periphery of the lobule was less affected than other parts; Carroll in 1905 made a similar observation, but Otto and Neumann in the same year asserted that islets of cells next to the central veins were the last to be destroyed. Boyce (1911) thought that necrosis was most marked in the central zone. In 1912 Rocha Lima described the midzonal necrosis as characteristic of the yellow fever liver, and this idea has been for many years the chief criterion for an anatomical diagnosis of the disease. As we have shown, it is quite fallacious to permit the diagnosis to rest upon this finding, for not only is midzonal necrosis found in other conditions, but it is frequently not to be distinguished in yellow fever. Nowadays, largely owing to the publications of Chiari (1925), Torres (1926), Couto and Rocha Lima (1929), and Hoffmann (1929), both the midzonal and the sporadic selectivity are recognized, but the essential characters of the hyaline necrosis are not sufficiently accentuated.

The earlier authors emphasized fatty changes above all other features of the yellow fever liver. Thus, Marchoux and Simond (1906) agreed with Sodré and Couto (1901) that yellow fever was virtually a "generalized steatosis." Wasdin (1898), Otto and Neumann, Carroll, Boyce, and Turnbull were all of the opinion that fatty degenera-

tion played the most conspicuous role. As regards interstitial hemorrhage, there are many contradictory statements. This may have arisen from the fact that cases of Weil's disease showing hemorrhage in the liver have found their way into the yellow fever literature. Our findings agree with Sodré and Couto, Rocha Lima, Chiari, and Hudson, who emphasize the absence of hemorrhage.

The French Commission under Marchoux (1903–1906) believed that the blood capillaries in the liver parenchyma were entirely collapsed by the swelling of the liver cells; they thought this brought about an obstruction in the portal circulation which could be held responsible for gastro-intestinal hemorrhages, epigastric pain, and even the anuria of yellow fever. Their idea is merely of historical interest for there is no real collapse of the sinusoids. Rocha Lima believed that the congestion sometimes observed in regions of concentrated necrosis results from a paralysis of the capillary walls in that location.

Difference of opinion exists upon the subject of wandering cell infiltration of the liver parenchyma in yellow fever. Here, again, Weil's disease probably accounts for some of the confusion. We agree with Elliott (1920) that exudative reactions are practically absent in all uncomplicated cases.

In June, 1928, Torres drew special attention to nuclear inclusion bodies in the livers of *M. rhesus* experimentally infected with yellow fever, and was the first to suggest a specific interpretation for them, though one of us (Klotz) had previously commented upon the same nuclear bodies in a personal communication to Dr. F. F. Russell (October, 1927), comparing them to herpes inclusions as described by Goodpasture. Torres has published several well illustrated articles setting forth the character of yellow fever inclusions as found in *M. rhesus*. He has described also (1929) the finding of inclusions in one of eight human cases, a result comparable to that of Hoffmann (1929), who demonstrated the change in one of four human cases.

Cowdry and Kitchen (1929 and 1930), have established the occasional presence of nuclear inclusion bodies in the liver of the human yellow fever subject. Their more recent work, which is exhaustive and elaborately illustrated, sets forth the nature and morphology of the nuclear inclusions as seen in this disease.

Quite recently Kuczynski and Hohenadel have published some experimental work claiming to have identified a bacillus as the causa-

tive agent of yellow fever, but the experimental lesions which they describe are not characteristic of this disease.

Summary

The gross appearance of the liver in yellow fever is characterized by the absence of distinctive changes such as are seen under the microscope.

Microscopically the liver tissue presents a non-inflammatory necrosis and necrobiosis of the parenchyma, unaccompanied by collapse of the tissue or interstitial hemorrhage. The outstanding change is a coagulative hyaline necrosis (the Councilman lesion), which does not attack the hepatic cells in an orderly fashion but occurs as a diffusely sprinkled lesion often most marked in the midzone (the Rocha Lima distribution). This specific necrosis is always preceded or accompanied by fatty degeneration and cloudy swelling. In the earliest stages it is characterized by the formation of dense acidophilic masses within the neutrophilic cytoplasm of the liver cells. Later, discrete, highly refractile, hyaline, globular bodies appear, often possessing a flattened pyknotic nucleus at the periphery of the mass. These bodies are usually honeycombed with fat vacuoles which they have incorporated. The process goes on to massive involvement of parenchymal tissue and ends in cellular disintegration, but no appreciable autolysis of the affected cell structures is seen in the acute stages.

The Kupffer cells suffer some damage, but not necrosis.

The vascular system, biliary channels, and stroma are uninvolved in the disease.

Specific nuclear inclusions were found in the livers of seventeen of nineteen *M. rhesus*, and twenty-three of ninety-three human cases of yellow fever. Their identification was attended with difficulties, but when their presence could be established the diagnosis was thereby facilitated.

The glycogen store of the liver is depleted in proportion to the severity of the lesion.

The liver lesions of yellow fever possess several features in common with the lesions of other virus diseases.

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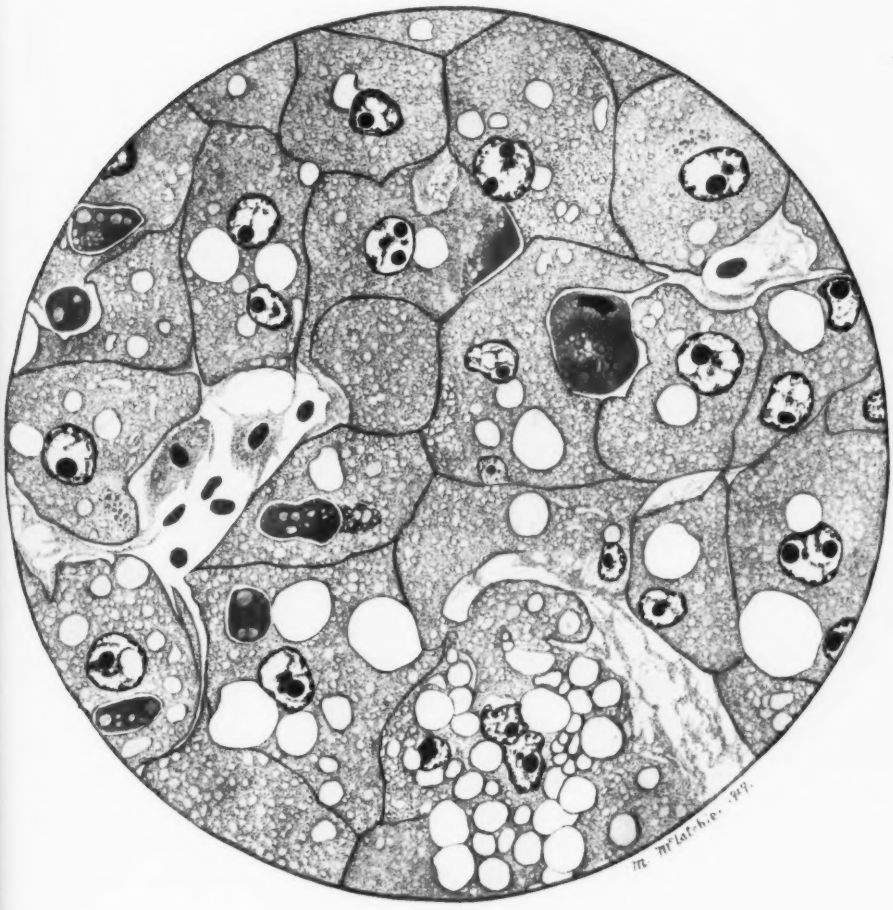
DESCRIPTION OF PLATE

PLATE 126

FIG. 1. Section of human liver (magnification about 1500). Showing (*a*) Councilman hyaline lesion, (*b*) granular degeneration (cloudy swelling), (*c*) globular and granular fatty vacuolization of cytoplasm, (*d*) pigmentation, (*e*) swelling of Kupffer cells, and (*f*) non-specific nuclear degeneration.



Klotz and Belt Pathology of Liver in Yellow Fever

REGENERATION OF LIVER AND KIDNEY FOLLOWING YELLOW FEVER *

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The present report is concerned with the study of liver and kidney tissues taken from six rhesus monkeys which had been experimentally infected with yellow fever at the laboratories of the West African Yellow Fever Commission of the Rockefeller Foundation at Yaba, Nigeria, in 1928. Each animal suffered a short but typical attack of the disease, manifesting symptoms of prostration, anorexia, fever, albuminuria and slight jaundice. Each recovered its usual health shortly after the cessation of fever, and in each case an active immunity was proved before the monkey was finally killed. The accompanying table indicates the individual histories, briefly.

Case Histories of Rhesus Monkeys Experimentally Infected with Yellow Fever

Monkey	Strain of virus	Incubation period	Fever	Post febrile period	Killed by
		days	*days*	*days*	
1	Asibi	2	4	16	Blow on head
2	A. S.	3	2	72	Ether
3	Asibi	5½	2	51	Blow on head
4	P.	5	3+	66	Blow on head
5	A. S.	7	2	54	Blow on head
6	I.	5	3	55	Blow on head

There is every reason to believe that these animals suffered appreciable damage to the liver and kidney during their attacks of yellow fever. Eighteen other monkeys, infected at approximately the same time, with the same viruses, and under the same conditions, died of the disease in periods ranging from two to eight days after the onset of fever. They showed, without exception, marked necrosis of the liver, sometimes amounting to total destruction of the parenchyma;

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation.

Received for publication June 10, 1930.

and the kidney in each case presented a severe tubular degeneration. The monkeys that recovered and those that died manifested similar clinical symptoms, except that in the latter the rise in temperature was of a higher and more constant character and the symptoms in general were of greater severity.

Assuming, then, that the six monkeys that recovered had sustained liver and kidney injuries comparable to those found in the fatal cases, the organs were examined with the express purpose of determining the final result of these injuries.

The autopsies revealed no unusual gross findings. Without exception, the livers and kidneys presented a healthy appearance, and showed no signs of antecedent injury. Microscopically there was an absence in all the preparations of the distinctive lesions associated with the acute phase of the disease, and scarring was not observed.

None of the livers showed increase in fibrous tissue or proliferation of biliary channels, such as is commonly seen following other types of liver injury. The lobules were of the usual size and structure, and the vascular channels were practically free from blood.

The liver cells possessed a more or less normal appearance. The nuclei were uniform, of the usual size, and well filled with chromatin, and they stained in a normal fashion. There was no indication of mitosis in any part.

In five of the six cases there was some degree of zonal differentiation. Thus, in three cases the cells of the portal zones were slightly swollen and more eosinophilic than those of other parts; the cytoplasm was finely granular and the adjacent sinusoids compressed. In the other two livers this same change was observed in the central vein areas, and the portal zones were occupied by a well marked fatty infiltration. It is possible that these zonal alterations may represent some functional readjustment to an antecedent zonal necrosis. On the other hand they may be related to an entirely irrelevant condition, such as, for instance, helminthiasis, which was present in practically all of the animals.

The liver of *M. rhesus* 6 was quite different in many respects from the livers of the other five monkeys of the series. There was no zonal differentiation. All parts of the sections showed a uniform rarefaction of the parenchymal cytoplasm and a marked edema of the cells. The sinusoids were compressed and the trabecular arrangement could not be made out. The swollen, hydropic cells were unusu-

ally clear in outline, and their closely packed arrangement lent a mosaic appearance to the sections.

Some minor changes of an indefinite character were found in the livers of all the monkeys of the series. The Kupffer cells were slightly more prominent than usual, both by virtue of their numbers, and because of the deep staining qualities of their nuclei. In several instances there appeared to be some distortion and irregular enlargement of the liver cords in the midzonal regions of the lobules. Sometimes there were four or five cells abreast in a single cord, suggesting an alteration in the original orderly arrangement of the tissue. The distortion was, however, unaccompanied by signs of karyokinetic activity or alterations in staining qualities, and it cannot be regarded as definite evidence of regeneration. The same appearance of distortion may be produced in normal liver tissue, if the microtome knife sections the lobule at an unfavorable angle. There was also in most instances slight lymphocytic infiltration of the portal sheath, a relatively insignificant lesion which occurs very commonly in monkeys that have not had yellow fever. Thus there is no obvious association between the present findings and the initial injury.

The kidneys of all the monkeys in the series presented a more or less uniform appearance, which did not deviate appreciably from the normal histological picture. The acute renal lesions associated with the fastigium of the attack and recently fully described by Magalhães, were absent. No casts were seen, no scarring nor increase in fibrous tissue and, with two exceptions, no evidence of inflammation. M. *Rhesus* 5 and M. *rhesus* 6 each presented an acute non-suppurative nephritis of a focal character, confined to the interstitial tissue. The process in both instances was of very recent origin, and apparently quite unrelated to the attack of yellow fever.

The glomeruli were not congested and the capillary tufts showed no cellular increase. The straight tubules possessed empty lumina and their epithelium was not altered.

The convoluted tubules were examined carefully, because these structures are most seriously affected in yellow fever. They contrasted in a normal fashion with the rest of the tissue, possessing the usual deep eosinophilic staining reaction. The cytoplasm was of the usual density, but finely granular. The cells were somewhat swollen and the margins of the lumina were often slightly frayed. There was no vacuolation nor desquamation of the epithelium, however, and

there were no collections of cellular débris in the tubules. Moreover, the nuclei were healthy in appearance and indicated that the apparent cloudy change present in the cytoplasm was not associated with a lowered vitality of the cells. On the other hand, there was no definite evidence of an increased vitality or vegetative activity such as one might interpret as a sign of recent regeneration. The nuclei in cross-sections of the tubules were not increased,* nor were there any of the epithelial giant cell formations which Oertel interprets as evidence of repair.

Discussion

It has been emphasized elsewhere (De Lamar Lectures 1927–1928), that cirrhosis of the liver does not follow yellow fever in man, and that a contracted kidney is likewise not a sequel of the disease. To this statement may now be added the weight of experimental evidence which shows that in the stage of repair, restitution of the affected organs is accomplished by complete regeneration of the functional tissues. The animals under discussion presented a practically normal histological picture of the organs except for certain irrelevant lesions. In liver and kidney alike, the restoration of the *status ante quo* was such that no sign remained to indicate the extent of regeneration or the manner in which it was accomplished.

The typical condition of the kidney in the acute stages of yellow fever corresponds in the main to a well known type of nephropathy, namely, the nephrosis of Volhard and Fahr, the "nephropathia degenerativa" of Aschoff, or more particularly the "bichloride kidney" of Elwyn. A distinction has long been made between this purely degenerative type and the inflammatory lesions of the kidney. Nephrosis is a condition met with commonly enough in acute toxemias, and it is generally recognized as a lesion which clears up completely when the toxemia passes off. The yellow fever kidney affords a very good example of a pure nephrosis, and subsequent complete regeneration is, therefore, not to be viewed as unusual or materially different from the sequence of events following nephrosis in other diseases.

The acute liver injury, on the other hand, while having several features in common with that of the kidney in yellow fever, is of a

* According to Ribbert the average normal number of nuclei is seven.

distinctive character, as we have been at some pains to show in a previous communication; and in consideration of the severe and extensive necrosis it is noteworthy that complete and scarless restitution of the liver takes place in cases of recovery. Clinical experience with yellow fever shows that it differs from similar pathological processes of equal intensity in the matter of freedom from sequelae in the liver. Various kinds of hepatorenal poisonings, as for instance, arsenic, phosphorus and carbon tetrachloride poisonings, and some toxemias of pregnancy, have a tendency to produce permanent liver damage, which not infrequently progresses into the fatal stages of acute yellow atrophy (Roman, 1927). Similarly, infective processes such as catarrhal jaundice and some obscure forms of hepatitis lead to marked scarring of the liver. In yellow fever, on the contrary, both clinical and experimental evidences tend to show that normal hepatic function is quickly restored in individuals that recover, and that these individuals never suffer cirrhosis of the liver or acute yellow atrophy as a consequence of their attack.

What are the factors which favor this peculiar power of regeneration? Undoubtedly the self-limiting character of the infection is of importance. Not only is the toxemia of relatively short duration, but there is no recurrence after the initial attack, no repetition of injury such as is commonly held to account for the sclerosing diseases. Yet in yellow fever the single injury is probably often of greater magnitude than the sum total of repeated injuries which in other diseases produce sclerosis. Thus the fact of a short period of injury is not a sufficient explanation. We must look to some peculiar features of the pathological process in yellow fever for an interpretation of the end results.

Inquiry into the absence of fibrous response in the yellow fever liver leads to a consideration of the character of the initial lesion. As we have pointed out elsewhere, yellow fever *per se* does not induce an inflammatory response. There is no exudative response to the injury and no appreciable migration of leucocytes to the damaged tissues. The Councilman necrosis of the liver, peculiar to this disease, is coagulative rather than autolytic, and since leucocytes are not attracted to the foci of necrosis, there is, presumably, no pouring out of proteolytic substances. The vascular channels, both extra- and intralobular, remain free from thrombi so that the circulation is not interrupted, and no digestion of tissue arises through infarction. It

is probably in consequence of these facts that rapid autolysis of the destroyed cells does not occur; no dissolution of tissue is to be found during the acute and fatal stages of the disease. The injury is thus qualitatively and quantitatively limited, and as a result only the parenchymal cells suffer from the destructive process. Not only is the stroma of the portal sheaths unaffected, but the slender intralobular connective tissue framework, which supports the trabeculae, remains intact and can be so demonstrated by Van Gieson staining technique even when necrosis of liver cells is most intense. The absence of scarring in the yellow fever liver is to be attributed, therefore, to an absence of injury or irritation upon the stroma, which in turn must be related in part to the non-inflammatory, non-autolytic character of the degenerative process, and in part to the maintenance of a normal blood supply.

The yellow fever liver then, provides a striking illustration of the point which Mallory emphasized (1911), that destruction of the parenchymal cells alone does not, of itself, stimulate connective tissue proliferation. Whipple and Sperry (1909), and Schultz, Hall and Baker (1923) have made the same observation in connection with chloroform poisoning. Before the process of fibrosis is set in motion, the injury must involve tissue elements other than parenchymal cells, and this is true of the kidney as well as of the liver. This view is somewhat at variance with that of Kretz (1905), MacCallum (1904), and Milne (1909), all of whom consider that cirrhosis arises in consequence of a primary destruction of liver cells.

We have indicated the conditions associated with the absence of fibrosis in the yellow fever liver. For a discussion of the factors which incite fibrosis in other diseases, the reader is referred to Mallory (1911), Pearce (1904, 1906), Muir (1908), Milne (1909), Opie (1910), Rolleston (1912), Herxheimer and Gerlach (1921), Schultz, Hall and Baker (1923), Hall and Ophüls (1925), Roman (1927), MacMahon and Mallory (1929), and MacMahon, Lawrence and Maddock (1929).

Our investigations of a large series of fatal cases of yellow fever in man and monkey show that active regeneration of liver and kidney tissues is practically at a standstill during the toxic phase of the disease. The series of animals that recovered, which we report here, shows however, that regeneration is complete within sixteen to seventy-two days after the cessation of fever. Therefore, the rep-

arative process must go on to completion within the ordinary period of convalescence.

In the case of the liver, it is not difficult to imagine how breaches of continuity in the parenchymal cords are bridged over by proliferation of liver cells which have survived the toxemia. The slender cylindrical stroma encasements of the trabeculae are preserved and the growing cells spread by direct extension inside of these limiting membranes to replace the cells which have been destroyed and absorbed. Thus, the original pattern of the tissue is restored. All investigators of repair in the liver agree that the chief rôle in the regeneration of the parenchyma is played by old, undamaged liver cells. MacCallum (1902) observed that "where well-differentiated liver cells still persist the new liver tissue is very simply produced by their mere multiplication by division, and the less highly differentiated gall-ducts take no part in the process, but remain quiescent in their subordinate position as conductors of the secretion of the liver cells." The much debated question as to whether liver cords may take their origin from biliary epithelium does not arise in connection with the present study, for, as we have seen, there is no proliferation of the bile ducts in the tissues under discussion. It is only when the supporting stroma has been damaged and stimulated to proliferation, as in the case of acute yellow atrophy and in the cirrhoses, that attempts at regeneration take the peculiar distorted form of pseudobile ducts or pseudotubules and nodular hyperplasia. This type of regeneration, fully reviewed by Hess (1913), Blum (1923), Roman (1927), and Fishback (1929), does not concern us in connection with yellow fever.

In the kidney, regeneration of the convoluted epithelium is no doubt accomplished largely by proliferation of cells which survive the attack. Islands of living cells always remain from which a new lining may originate. In certain cases of extensive necrosis, it is probable also that the cells of the straight tubules may grow in to replace the destroyed secretory epithelium, assuming its morphology and its specialized function. As in the case of the liver, the intact basement membranes which remain unaltered in the disease orientate the new cells into the old pattern, as proliferation and extension take place. The functionless regeneration phenomena described by Oertel (1909) in chronic nephritis, and by Podwyssozki (1887), Ribbert and Peipers (1895), Thorel (1907), and Pearce (1909)

in partial extirpation experiments, as might be expected, are not to be found in the yellow fever kidney.

Summary

Six rhesus monkeys which had recovered from experimental yellow fever showed complete and scarless regeneration of the liver and kidney. This bears out clinical evidence that neither cirrhosis of the liver, nor contracted kidney follows yellow fever in man.

Special attention is directed to the sequence of events taking place in the liver. Except in cases of chloroform poisoning, liver damage of equal magnitude rarely occurs without producing some scar formation. The yellow fever liver proves that destruction of parenchymal cells alone is not a sufficient stimulus to induce replacement fibrosis.

The absence of fibrosis in the liver and kidney is due to a peculiar immunity which the stroma structures manifest toward the yellow fever injury; there is no stimulation of connective tissue elements during the acute stage of the disease. The reasons for this are, we believe, related to the non-inflammatory, non-autolytic character of the acute pathological process, and to the absence of thrombosis in the small parenchymal vessels.

Regeneration originates in islands of parenchymal cells which have survived the attack, and quickly restores the tissues to their original state.

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THE NATURE OF FOWL-POX VIRUS AS INDICATED BY ITS REACTION TO TREATMENT WITH POTASSIUM HYDROXIDE AND OTHER CHEMICALS*

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Recent experiments,[1,2] have given additional morphological evidence that the inclusion bodies found in the infected epithelial cells of a fowl-pox lesion represent colonies of a minute microörganism (Borrel bodies[3]) embedded in a ground substance of lipoproteid composition. Following this work, the highly infectious nature of isolated inclusion bodies was demonstrated by Woodruff and Goodpasture.[4] In further experiments,[5] it has been shown that a fraction of an inclusion will produce a typical fowl-pox lesion upon inoculation. This demonstration of the high degree of infectiousness of the inclusion bodies, and the definite morphology of their Borrel body components aroused our interest in the work of previous investigators which led them to believe that the virus of fowl-pox or pigeon-pox is a non-living agent. In particular, the experiments of Sanfelice attracted our attention since they are so frequently quoted in the literature as indicating the non-viability of at least one of the viruses. For this reason Sanfelice's work was repeated with care and will be described in some detail.

Sanfelice used pigeon-pox for his experiments. The virus of pigeon-pox is infectious for the chicken and induces a lesion identical with that of the strain of virus indigenous to fowls. Sanfelice,[6] by treating the virus of pigeon-pox with 1 per cent potassium hydroxide, claims to have extracted a nucleoproteid toxin which would produce typical lesions. His technique was to grind thoroughly a piece of the fresh tissue from a pigeon-pox lesion and to add 1 per cent potassium hydroxide amounting to two or three times the weight of the tissue. After standing four to twenty-four hours and longer with the potassium hydroxide, the material was filtered through a piece of linen and the filtrate treated with twice its volume of 1 per cent

* Received for publication July 26, 1930.

acetic acid. The resulting precipitate was then washed and inoculated. Such inoculations always produced typical lesions, while portions of the tissue treated with potassium hydroxide, washed, and inoculated without acetic acid treatment, gave uniformly negative results.

Since the method of extracting tissue with potassium hydroxide and then precipitating with acetic acid is one employed in preparing nucleoproteids, Sanfelice assumed that he had isolated a nucleoproteid agent responsible for the disease. This agent, according to his hypothesis, is a toxic material which has the properties of an acid, being inactive or neutralized when combined with potassium hydroxide, but readily liberated upon the addition of an excess of acid. Such a non-living, toxic nucleoproteid, he proposed, is elaborated by the affected cells, and, upon transmission to other cells, causes these in turn to elaborate more of the same agent.

Sanfelice's argument that no living agent could be responsible for "takes" at twenty-four hours is based upon earlier work, which, he says, was corroborated by Burnet.* In 1897, at a time when he held that the agent of pigeon-pox was a blastomyces,[9] Sanfelice tested the toxic effects of several chemicals and disinfectants upon the virus. Among these were potassium hydroxide, acetic acid, and phenol. He found that the virus was killed after five minutes in 0.5 and 1 per cent potassium hydroxide, 1 per cent acetic acid, and 0.5 and 1 per cent phenol. In these experiments, however, Sanfelice employed a very difficult technique. Silk threads, immersed in pigeon-pox material, were dried at 37° C, then immersed in 1 per cent potassium hydroxide for five minutes, washed in sterile water, and finally scraped to obtain material for inoculation. This procedure would, we believe, be difficult to perform without many chances for the complete loss of the virus; and the results should not be used as a basis for comparison with other experiments in which larger amounts of infected tissue were employed.

Sanfelice has by no means gained universal acceptance of his hypothesis. Friedberger[10] in 1918 found no evidence that virus "inactivated" by potassium hydroxide could be reactivated by acetic acid. He reported that pigeon-pox virus in dilute suspension was

* Burnet[7] merely refers to a paper by Reischauer[8] in which Sanfelice's original work is quoted.

killed, as was *B. prodigiosus*, after five minutes treatment with 1 per cent potassium hydroxide. He then employed Sanfelice's technique and obtained typical lesions after treatment of virus fourteen hours in 1 per cent potassium hydroxide, not only with inoculations of the "nucleoproteid" precipitate, but with control inoculations of filtrate treated with potassium hydroxide alone. Friedberger stated that Sanfelice's linen filter let through large particles of tissue which no doubt resisted the action of 1 per cent potassium hydroxide longer than the dilute suspension that he himself used in comparing its viability with that of *B. prodigiosus*. Sanfelice still upheld his nucleoproteid hypothesis, however, and in 1927 produced another paper [11] in which the refutation of Friedberger was attempted.

In our own experiments to test the effect of 1 per cent potassium hydroxide on the virus, dried rather than fresh scabs from the lesion of fowl-fox were used, and a filter of absorbent cotton rather than linen. Otherwise Sanfelice's technique was followed exactly. The dried scabs were ground in a mortar with distilled water or saline, and the resulting suspensions were found to contain virus in such concentration that, when inoculated on a chick, positive results were obtained in dilutions as great as 1:1000. The suspensions were then mixed with equal quantities of 2 per cent potassium hydroxide, and these suspensions of fowl-pox material in 1 per cent potassium hydroxide were allowed to stand at room temperature for periods of four and ten hours. After such treatment, part of the material was filtered through absorbent cotton, and precipitated with twice its volume of 1 per cent acetic acid. The precipitate was then washed and inoculated. Inoculations after four hours produced only small lesions while those after ten hours were negative. At the same time, some of the sediment from the potassium hydroxide suspension, thrown down by centrifuging and then carefully washed, was found upon inoculation to produce massive lesions after both four and ten hours. These experiments seemed to point directly to the size of the particles as being an important factor in the protection of virus from the action of the potassium hydroxide, since in the centrifuged sediment the particles were obviously larger than those in the filtrate. We noted also in an experiment with unfiltered material, that inclusion bodies were present in the tissue. After treatment of this material with 1 per cent potassium hydroxide for twelve and twenty-four hours, sediment freed from the potassium hydroxide by merely

washing, as well as by precipitation with acid, produced typical lesions upon inoculation.

Following these experiments in which the effect of potassium hydroxide was determined on comparatively large particles of tissue, an attempt was made to obtain suspensions which might contain only that most minute element of the fowl-pox lesion, the Borrel body. A series of experiments was started using the dried scabs from fowl-pox lesions. These were carefully ground in distilled water and then the larger particles were thrown down by centrifug-

TABLE I

Effect of 1 per cent Potassium Hydroxide on Finely Divided Suspensions of Virus

Virus Suspensions		Time at which Virus was Tested				
Infectious at dilution of	Dilution employed in KOH experiments	2 min.	½ to 4 hrs.	9 hrs.	16 hrs.	19 to 24 hrs.
1:1000	1:2	..	..	..	..	−
1:1000	1:2	..	..	..	..	+
1:1000	1:2	+++	..	..	..	−
1:1000	1:2	+++	..	..	..	−
1:1000	1:2	..	..	..	..	−
1:100	1:2	+	..	..	..	−
1:2000	1:2	+++	+++	..	..	..
1:2000	1:2	..	+++	..	..	..
1:2000 *	1:2	+++	+++	..	..	..
1:2000 *	1:2	..	+++	..	..	..
1:2000 **	1:2	..	+++	..	..	−
1:2000 **	1:2	+++	+++	..	..	..
1:2000 **	1:2	+++	..	+++	++	+

* = filtered
** = filtered (Whatman 42)
+++ = massive lesion
++ = several nodules
\+ = one or two nodules
− = negative

ing fifteen to twenty minutes at low speed. The fine supernatant suspension was decanted, and in some instances filtered through filter paper. In one or two experiments, especially fine filter paper (Whatman 42) was used. While we cannot say definitely that the resulting filtrate was a Borrel body suspension, yet we are certain that the precaution taken excluded any large inclusions, and that, in addition to Borrel bodies, only very small or fractional inclusions were present. In order to test the virulence of this filtrate, part of it was used for inoculation at various dilutions. The rest was mixed with equal quantities of 2 per cent potassium hydroxide to give a

1 per cent concentration, and allowed to stand at room temperature. With this finely divided material, the only means of freeing the virus from potassium hydroxide was to neutralize with acetic acid and wash. This we did, finding that the protein precipitate which followed addition of acid carried down with it any active virus, while the supernatant fluid was generally innocuous.

Table I shows that virus inoculated after two minutes treatment produced massive lesions. Similar lesions were obtained up to four hours, and in one instance at nine hours (see Fig. 1). The length of resistance probably varied with the fineness of the suspension. Two lesions of one or two nodules were obtained in the nineteen to twenty-four hour period (see Fig. 2), but the majority of these inoculations were negative.

The important point in the above experiments is the fact that the suspension of virus showed evidence of being progressively destroyed — the longer the treatment, the smaller the lesion. There was no suggestion whatever that virus once inactivated could be "reactivated" by means of acetic acid. A few experiments, in which fresh tissue from the fowl-pox lesion was used instead of the dried tissue, indicated that resistance might be somewhat longer with fresh material than with the dried. However, the fresh material too showed evidence of being progressively diminished in virulence rather than of having any ability to be "reactivated" at full strength after long periods of treatment.

Since the above experiments indicated that the size of the particles which were treated with potassium hydroxide was an important factor in determining the resistance of the virus, it seemed logical to make use of the fowl-pox inclusion bodies in further work. As has been demonstrated,[4] these inclusions are highly infectious and can be obtained free from cellular material by tryptic digestion. Accordingly, suspensions of inclusion bodies were obtained by tryptic digestion. The inclusions were removed by centrifuging and were washed in saline or distilled water. Equal volumes of inclusion body suspensions and 2 per cent potassium hydroxide were mixed in order to obtain suspensions of inclusions in 1 per cent potassium hydroxide. These were treated for periods of from one to six days, the material being transferred to fresh sterile test tubes every twenty-four hours to insure uniform action of the chemical. At the end of these treatments, the suspensions were carefully removed,

placed in sterile test tubes and centrifuged. The inclusion sediment was washed with sterile saline, and inoculated either into feather follicles or on defeathered, scarified areas.

Table II shows that inclusions will resist the action of 1 per cent potassium hydroxide for from one to five days, the size of the lesion produced diminishing with length of treatment. The massive lesion produced by bodies treated twenty-four hours is shown in Fig. 3. Fig. 5 shows the smaller lesions produced by bodies treated seventy-two hours. It was found that different specimens varied in their ability to resist the action of 1 per cent potassium hydroxide. In-

TABLE II

Virucidal Effect of 1 per cent Potassium Hydroxide on Inclusion Bodies

Time in KOH		16 to 24 hrs.	2 days	3 days	4 days	5 days	6 days
No. of inoculations		23	11	13	8	5	2
No. of positives.....		23	8	8	1	2	0
Type of Lesion..	+++	23	..	..	..	..	..
	++	..	6	1	1	..	..
	+	..	2	7	..	2	..

+++ = massive lesions. ++ = several nodules. + = one or two nodules.

clusions obtained from a fowl-pox lesion more than ten days old seemed more fragile and more readily damaged than those from a seven- to ten-day lesion. In these experiments it will be noted that mere washing after potassium hydroxide treatment was sufficient to prepare bodies for inoculation. While neutralization of the potassium hydroxide with acetic acid was effective, the simple washing of the inclusions with saline gave just as large lesions after just as long periods of treatment as did the acetic acid method. After treatment for a sufficient length of time, varying from two to six days, neither washing nor neutralizing with acetic acid was effective in "reactivating" the virus.

Since recent work points to the Borrel body as the probable etiological agent of fowl-pox, it seemed of interest to determine whether Borrel bodies could still be identified in inclusions which had been proved infectious after a long period in potassium hydroxide. It was found that after treatment with potassium hydroxide, the physical characteristics of the inclusion bodies were altered. A jelly-like substance developed about each body causing groups of bodies

to stick together. Within this film, the definite outline of the compact inclusion was apparent. When the usual technique [5] of drying out of distilled water was applied to these inclusions, they failed to break up and liberate their Borrel bodies. Moreover, in stained preparations, such inclusions appeared to have hard, incrusted surfaces, and it seemed that some further means was necessary to break up the inclusions after potassium hydroxide treatment. After some experimenting, it was found that rupture of the inclusions could be brought about mechanically by means of a small glass point. Fig. 8 shows a smear of Borrel bodies obtained from inclusions which had been in 1 per cent potassium hydroxide for twenty-four hours, then washed in distilled water and scratched with a fine glass point. Fig. 9 shows an inclusion stained after such treatment. One side of the inclusion has been ruptured, and the contents have poured out in fan-shape. Characteristic Borrel bodies are shown at the edge of the mass where the bodies are more widely dispersed. In Fig. 10, the same inclusion is shown with the broken edge of the surface of the inclusion in focus. The granular structure of the extruded contents and of the material remaining within the inclusion may be seen. The broken edge of this inclusion gives one the impression that the outer surface is hardened and shell-like. Such an incrustation of the inclusions may be responsible for their long survival in potassium hydroxide, though no data have been obtained as to which component of the lipoproteid ground substance of the inclusion reacts with the potassium hydroxide to produce this hardening.

A possible explanation for the "reactivation" obtained by Sanfelice after twenty-four hours in potassium hydroxide is that the linen filter which was used by him allowed larger particles of the diseased tissue to pass than did our absorbent cotton filter, or fine filter paper. These larger particles were, we judge, responsible for the successful inoculations rather than any nucleoprotein extract. This possibility has been suggested also by Friedberger, but he did no further work to determine the actual viability and structure of such large particles in potassium hydroxide. In the grinding of fresh material it was found impossible to break up completely all the masses of inclusion bodies present in the diseased tissue. Many inclusions are freed by grinding but are not further injured by ordinary grinding methods. Furthermore, digested inclusions pass readily through a linen filter such as Sanfelice described. While the

filtration of ground material in 1 per cent potassium hydroxide is slower, due to the viscidness of the suspension, still the possibility of small inclusion bodies passing through the linen filter is obvious, and, as we have shown, these may be still viable.

In a further series of experiments, an attempt was made to extract a toxic nucleoproteid from inclusion bodies, such as that described by Sanfelice. Since inclusions are so highly infectious, a specific toxin in the sense of Sanfelice might be expected to be present in

TABLE III

Toxin Theory Tested with Inclusion Bodies

	Filtrate of supernatant fluid infectious at dilution of	Filtrate of supernatant fluid 16 to 24 hours in KOH	Sediment of inclusion bodies 16 to 24 hours in KOH
A	1:5	−	+++
	1:2	−	+++
	1:2	−	+++
	1:2	−	+++
	1:2	−	+++
	1:2	−	+++
B		−	+++
		−	+++
		−	+++

+++ = massive legion.
− = negative.

quantity within them. Saline suspensions of the digested bodies were made as previously described. These were allowed to stand about half an hour in order to obtain in the saline some active virus, free from inclusions. The suspension was then centrifuged and the top portion of the supernatant fluid filtered. This filtrate contained very weak virus, active in dilutions not greater than 1:2 to 1:5. The weak filtrate and the inclusion body suspension were treated separately with 1 per cent potassium hydroxide for long periods, sixteen to twenty-four hours. It should be stated that the potassium hydroxide and acetic acid were added to proportionate parts of the

weak virus suspension in such a percentage that the total dilution of this fluid was no greater than 1:2. After neutralization, the filtrate was inoculated. Results with the filtrate after potassium hydroxide and acetic acid treatment were uniformly negative, while inclusion bodies which were merely washed free of potassium hydroxide but were not treated with acetic acid always produced massive lesions (see Table III–A and Fig. 6).

The above experiment seemed fairly conclusive that the small amount of active virus present in a suspension free from inclusions is not a nucleoproteid toxin, for otherwise it could, according to Sanfelice's hypothesis, be reactivated by acetic acid treatment. In this experiment, however, we did not allow for the possibility that potassium hydroxide might be more effective than saline in extracting such a toxin from the inclusions. Therefore, we again prepared suspensions of digested and washed inclusions, placed them in 1 per cent potassium hydroxide, and after one-half hour, centrifuged to throw the inclusions to the bottom in order that they should not become mixed with the supernatant fluid and confuse the results. The whole amount of material was then allowed to stand with the potassium hydroxide for twenty-four hours. In this way any hypothetical nucleoproteid toxin present in the inclusions could be dissolved into the supernatant fluid. After twenty-four hours, portions of the supernatant fluid were filtered, neutralized with acetic acid, and inoculated. Table III–B shows that in three experiments this filtrate was inactive, while the sediment of inclusion bodies, washed only, produced massive lesions. This we judged, was sufficient evidence that no toxic product could be extracted, by Sanfelice's method, from inclusions, highly infectious as they are. In all respects, inclusions respond to potassium hydroxide treatment by a progressively diminishing strength, being capable of resisting the action of 1 per cent potassium hydroxide for several days, but once inactivated, no treatment with acetic acid is capable of "reactivating" them. We have been able to obtain no evidence in support of the nucleoproteid toxin theory.

In a paper [11] which appeared in 1927 refuting Friedberger's work, Sanfelice cited experiments in which carefully ground, diseased tissue was treated in one case with potassium hydroxide followed by acetic acid, and in the second case with acetic acid followed by potassium hydroxide. His inoculations made following the first procedure

were positive and, following the second procedure, negative. According to his own description, however, his technique was varied slightly in the two cases. In the first case the ground material was mixed with saline, filtered through ordinary filter paper and the filtrate mixed with 2 per cent potassium hydroxide. In the second case the ground material was mixed directly with 1 per cent acetic acid and this mixture filtered. The results which we have obtained in repeating this experiment indicate that the direct addition of acetic acid to the ground material forms a sticky mass which does not filter as readily as the saline suspension. It would seem, therefore, that Sanfelice's reported results are due to the passing of copious virus when the saline suspension was filtered, while the major portion of the virus was withheld from the filtrate when the sticky acid suspension was filtered.

In the course of this work with potassium hydroxide and virus, it was noted that inoculations made without first removing the potassium hydroxide showed severe scabbing. Tests were made to determine the action of 1 per cent potassium hydroxide alone on chicken epithelium. Epithelial tissue, treated with a single application of 1 per cent potassium hydroxide and excised after twenty-four hours, shows, in stained sections, that the epithelial cells have been completely destroyed (see Fig. 7). Experimental inoculations with active virus in potassium hydroxide have in several instances produced no lesions, and, in other instances, it has been noted that smaller lesions are produced than with an equivalent amount of virus from which the potassium hydroxide has been removed (see Figs. 4 and 3). Thus, destruction of epithelial cells may be the cause of negative results in experiments where the potassium hydroxide is not removed, since epithelial cells are necessary for the proliferation of the virus. Possibly the existing disagreement as to potassium hydroxide effects may be due in part to insufficient control of this factor.

A few further experiments on the virucidal action of chemicals have been carried on. In two experiments inclusion bodies were found still viable after two hours in 1 per cent phenol, in contradistinction to Sanfelice's report that the virus is killed after five minutes in 0.5 per cent and 1 per cent phenol.* The discrepancy here

* In a recent paper Kligler [12] reports that fowl-pox virus remains viable after fifty days in 0.25 per cent phenol.

may well be due to the different form of the virus — in one case inclusion bodies and in the other case finely ground material dried on silk threads. Our results indicate that the virus, when protected in the intact inclusion body, is very resistant to the chemical agents we have used.

Extraction of dried virus with alcohol, followed by ether, and with ether alone, for periods of one-half to one hour does not destroy the activity of the virus. Virus treated thus has proved active in dilutions as great as 1:1000. Inclusions dried and treated with ether for one-half hour kept their form and activity, though fat stains showed that most of the lipoid had been removed.

Discussion

Publications of Sanfelice,[6, 11] describe a technique for the isolation of a toxic nucleoproteid from the infected epithelium of a pigeon-pox lesion. Such a toxin he claims is capable of reproducing the typical lesion. After treatment of virus with 1 per cent potassium hydroxide, Sanfelice's technique requires the addition of 1 per cent acetic acid to "reactivate" the virus. Since this procedure is the usual one for the isolation of nucleoproteids, Sanfelice considered the activity of virus following treatment with potassium hydroxide to be due to the extraction of a toxic nucleoproteid. We have found that removal of the alkali by merely washing the tissue which has been treated with potassium hydroxide, is just as effective in obtaining active virus as is the method of precipitating with acetic acid. This work corroborates Friedberger's experiments.[10] The active material obtained after treatment with potassium hydroxide is therefore not necessarily a nucleoproteid derivative. Furthermore, after a sufficient length of treatment with potassium hydroxide, the virus becomes completely inactive, and cannot be "reactivated" by any method.

Thus our experiments lead us to make an interpretation, differing from that of Sanfelice, concerning the nature of the virus. Sanfelice considered that the virus had the properties of an acid, being inactive or neutralized when combined with potassium hydroxide, but readily liberated upon the addition of an excess of acid, while we have found that the virus is not inactivated immediately upon the addition of 1 per cent potassium hydroxide, but survives for a defi-

nite period of time. This period varies, depending upon the physical state of the virus. Suspensions of very finely divided material, we found, were progressively destroyed, being diminished in strength after several hours and completely inactive after twenty-four hours. Inclusion bodies, on the other hand, will resist the action of 1 per cent potassium hydroxide for from one to five days. This greater resistance of the inclusions is due, we judge, both to the great concentration of virus within them and to the protection offered by their lipoproteid supporting substance. Moreover, stains of inclusions which were proved to be still viable after twenty-four hours treatment with potassium hydroxide show that Borrel bodies are still present.

In attempts to extract a nucleoproteid toxin from inclusion bodies, freed from cellular material by digestion, we have found no evidence of the existence of a transmissible toxin. There is very little free virus in the fluid portion of a saline suspension of digested inclusions, and none if each inclusion is washed separately.[4] The small amount present in our suspensions never survived twenty-four hours treatment with 1 per cent potassium hydroxide, whereas inclusion bodies thus treated did not lose their infectiousness for a much longer period. This result indicates that the active virus of an inclusion is an integral part of the inclusion and not superficially adsorbed by it. If the virus were held merely on the surface of inclusion bodies, inclusions might be expected to lose their activity in potassium hydroxide as rapidly as free virus.

The fact that inclusions remain viable after long periods in potassium hydroxide, this viability being presumably dependent upon the survival of materials within the inclusion, together with the demonstration of the presence of Borrel bodies in inclusions after twenty-four hours treatment with potassium hydroxide, supports the evidence of Goodpasture,[1, 2] and Woodruff and Goodpasture,[4, 5] that the inclusions of fowl-pox are colonies of a minute microörganism, the Borrel body.

Summary

1. Fowl-pox virus in suspensions of finely divided material is rendered inactive after a period of four to twenty-four hours in 1 per cent potassium hydroxide. In the form of inclusion bodies, however, the virus is found to be infectious, in diminishing strength, after treatment with potassium hydroxide for as long as five days.

2. No evidence has been found for the existence of a nucleoproteid toxin, such as that described by Sanfelice, either in scabs of the fowl-pox lesion or in digested inclusion bodies.

3. The destructive action of 1 per cent potassium hydroxide on normal epithelial cells of the chick is shown.

4. The presence of Borrel bodies in inclusions which have been proved infectious after remaining twenty-four hours in 1 per cent potassium hydroxide has been demonstrated.

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DESCRIPTION OF PLATES

PLATE 127

FIG. 1. Massive fowl-pox lesion produced by inoculation of precipitate from Borrel body suspension after nine hours in 1 per cent potassium hydroxide.

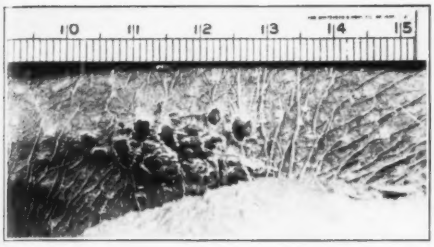
FIG. 2. Two small nodules on left edge of scar show the diminished infectiousness of the precipitate from a Borrel body suspension after nineteen hours in 1 per cent potassium hydroxide.

FIG. 3. Massive fowl-pox lesion produced by inoculation of inclusion bodies after twenty-four hours in 1 per cent potassium hydroxide.

FIG. 4. Result of the inoculation of same preparation of inclusions as used in Fig. 3, but without removal of the alkali.

FIG. 5. Small lesion produced by the inoculation of inclusion bodies after seventy-two hours in 1 per cent potassium hydroxide.

FIG. 6. The massive lesion on the right was produced by the inoculation of inclusion bodies after twenty-four hours in 1 per cent potassium hydroxide. The non-infected scar within the square on the left shows the negative result obtained by inoculation of free virus suspension after treatment for the same period.



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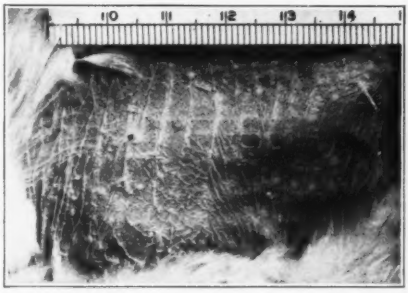

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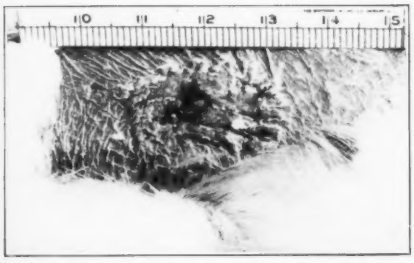

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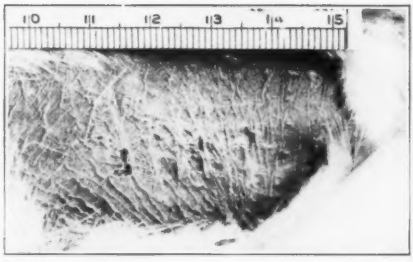

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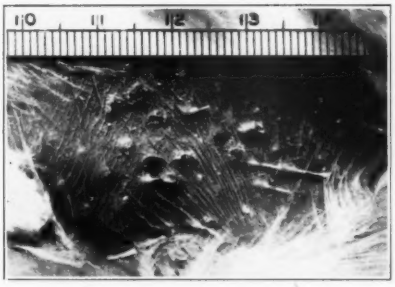

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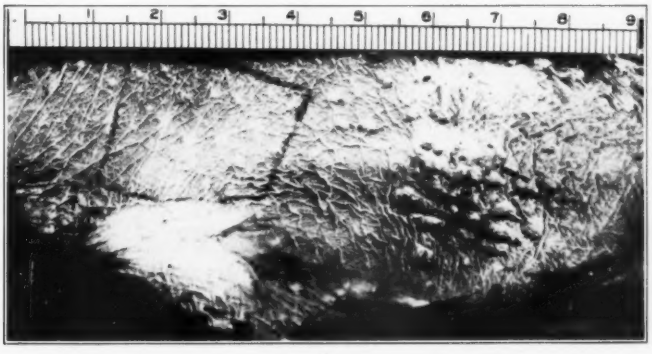

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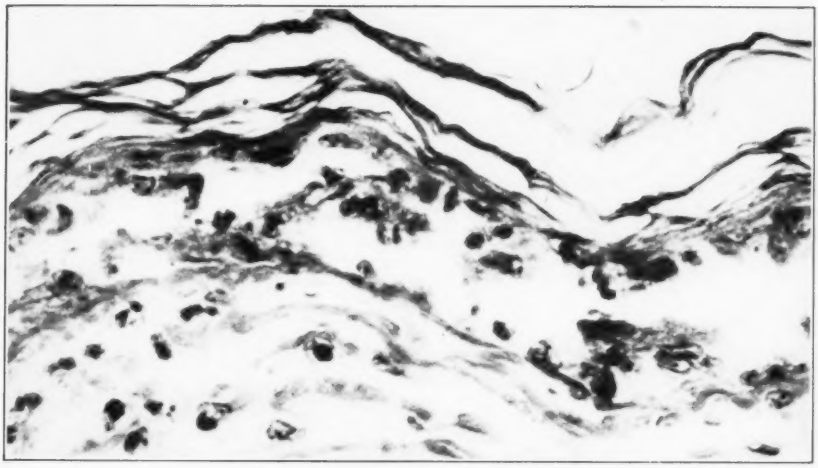
PLATE 128

FIG. 7. Complete destruction of epithelial cells of skin of chick after short application of 1 per cent potassium hydroxide.

FIG. 8. Borrel bodies from inclusions which had remained twenty-four hours in 1 per cent potassium hydroxide. Morosow's stain. Photomicrograph taken with blue light. × 1860.

FIG. 9. An inclusion body which had remained twenty-four hours in 1 per cent potassium hydroxide, mechanically ruptured to free the Borrel bodies. Morosow's stain. Photomicrograph taken with blue light. × 1860.

FIG. 10. Same ruptured inclusion as shown in Fig. 9 with focus on outside surface of body. Morosow's stain. Photomicrograph taken with white light. × 1860.



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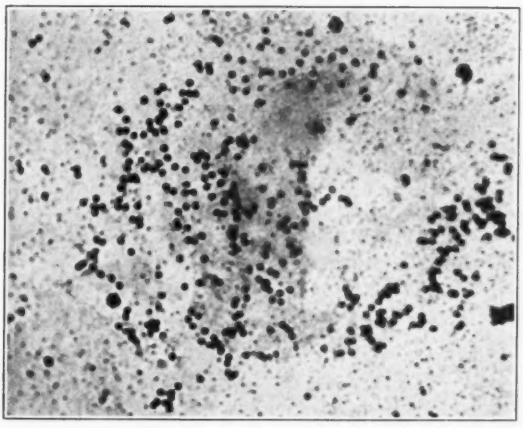

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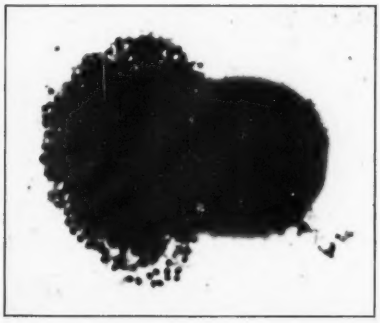

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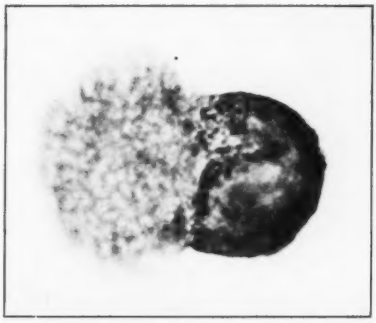

10

THE RELATION OF THE VIRUS OF FOWL-POX TO THE SPECIFIC CELLULAR INCLUSIONS OF THE DISEASE *

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The nature of cellular inclusions in virus diseases has been for years the subject of controversy. When first observed in fowl-pox, for example, the inclusions were thought to be protozoa which caused the lesion in which they appeared.[1] With the first experiments on filtration, however, any idea that the entire inclusion body represented a single protozoan parasite had to be abandoned on account of the obvious impossibility of any such structure passing intact through the fine pores of a filter.

Some of the more recent workers have not only abandoned the protozoan hypothesis, but have swung to the other extreme of relegating the inclusion bodies to the class of cellular degeneration products.[2,3] That the inclusion bodies of fowl-pox, at least, are indeed something other than mere products of cellular degeneration was demonstrated in a recent publication from this laboratory.[4] It was shown that upon subjecting a fowl-pox lesion to tryptic digestion the inclusion bodies resist the action of the trypsin, while the epithelial cells of the lesion are completely destroyed. The inclusion bodies, thus liberated from their host cells, could then be washed in sterile saline until the fluid immediately surrounding them was innocuous, while a single inclusion body, when inoculated into the feather follicle of a normal chick, produced a characteristic fowl-pox lesion. This demonstration of the infective nature of the inclusion bodies of fowl-pox gives rise to questions concerning the make-up of the inclusions, and especially the question as to what portion or portions of the inclusions, are responsible for the transmission of the disease.

THE COMPONENT PARTS OF THE INCLUSION BODIES

Since the observations of Borrel [5] in 1904, it has been known that smears of the fowl-pox lesion show innumerable minute, coccoid

* Received for publication July 26, 1930.

† The initial work on this paper was done while a Fellow in Medicine of the National Research Council.

structures 0.25 of a micron in diameter. These structures have been called Borrel bodies, after their discoverer. Borrel suggested that the inclusions of fowl-pox might be made up of these minute coccoid structures, and in 1906 Burnet [6] demonstrated in sections of the fowl-pox lesion stained in one case by Giemsa's method and in the other case by Loeffler's flagella stain, that the inclusions and the "micrococcal masses" belong to the same cells. In 1928 it was shown by Goodpasture [7] that the inclusion bodies of fowl-pox, when suspended in saline, appear as compact, hyaline structures, but that upon being washed and suspended in distilled water the same bodies swell markedly and develop vacuoles in which may be seen minute granules dancing about in rapid Brownian motion. These granules, as demonstrated by crushing an inclusion body on a slide, were shown to be Borrel bodies.

The marked change which occurs in fowl-pox inclusion bodies upon immersion in distilled water is shown in Figs. 1 and 2. In Fig. 1 may be seen eight inclusions in saline while Fig. 2 shows the same eight inclusions at the same magnification thirty minutes after distilled water had been substituted for the saline. There has been an increase in the linear dimensions of the bodies of one-third to one-half, indicating a volume increase of two to three times. One of the swollen inclusion bodies is shown under higher magnification in Fig. 3. The large vacuoles may be seen distinctly. In these, in the fresh preparation, the Borrel bodies could be seen in rapid Brownian motion. Fowl-pox inclusions, therefore, are composed of a semipermeable ground substance in which the Borrel bodies are embedded. This ground substance is thought to be a lipoproteid in composition.[8]

While working with inclusion bodies which had become swollen in distilled water, it was observed that if the water be allowed to dry, the inclusions, at the moment the water leaves their surface, will be torn into fragments with an abruptness almost explosive in character. The breaking up of the inclusions in this fashion we presume to be due to the force of surface tension, which is relatively enormous when acting upon such minute structures. This method of tearing apart inclusion bodies was found to be ideal for demonstrating the constituent parts of the bodies. Single bodies were isolated and washed, using a capillary pipette and the Chambers microdissection

apparatus. Upon allowing such a body to break up, a smear was obtained which showed the individual Borrel bodies and in which one could know with certainty that there were included only the Borrel bodies belonging to that particular inclusion. Such a smear of a single inclusion body is shown in Fig. 4. The Borrel bodies are the minute round structures appearing sometimes singly, sometimes in diploid form, and again in chains or masses. Under higher magnification the black areas in this photograph can be resolved into closely grouped masses of Borrel bodies embedded in the ground substance of the inclusion.

With the stained preparations of individual inclusion body smears it has been possible to get a fairly accurate idea of their enormous content of Borrel bodies. Actual counts have been made from photographic prints in which the Borrel bodies were magnified 1000 diameters. In each instance one thousand adjacent Borrel bodies were counted and from that count the total number in the smear was estimated. Using this method six thousand Borrel bodies were found in one inclusion, in another twenty thousand, and in a third ten thousand. All of these estimates were conservative and it is believed that a more accurate count would have revealed more, rather than fewer, Borrel bodies.

In order to get an idea of the relative size of the Borrel bodies, hemolytic streptococci from a culture were introduced in a preparation of inclusion bodies in distilled water. A portion of the resulting smear is shown in Fig. 6. The streptococci, on account of their greater size, are in a focal plane slightly different from that of the Borrel bodies. For this reason the streptococci are in the sharpest focus in the lower and right-hand portion of the picture where the Borrel bodies are out of focus.

The stain used in this preparation was Morosow's modification of the Fontana-Tribondeau method [9] and consisted essentially of a mordant of tannin followed by a silver nitrate solution. On account of the mordant the streptococci in Fig. 6 appear much larger than streptococci from the same culture stained by Gram's method. Presumably the Borrel bodies appear similarly enlarged, due to the mordant. By Morosow's method the streptococci and Borrel bodies stain a deep brown or black. The photograph was taken using a blue light.

The Inoculation of Small Portions of Inclusion Bodies

Following our successful experience with the inoculation of isolated and washed inclusion bodies in chicken-feather follicles,[4] numerous attempts were made to disperse the individual inclusion bodies in some fashion which would allow the inoculation of their fractional parts. On account of the extreme tenuousness of the lipoproteid material in the bodies, all of our efforts in this direction were fruitless until the method just described of allowing the bodies to dry out of distilled water was devised. With this method at hand it seemed that portions of the inclusion body smear might readily be scraped up for purposes of inoculation. Accordingly, single inclusion bodies which had been freed of cellular material by tryptic digestion, were isolated, using a capillary pipette in the Chambers microdissection apparatus. The single inclusion was then transferred to a pool of sterile distilled water on a large-sized coverslip and thoroughly washed by sucking up the major portion of the distilled water in a pipette and then replenishing the pool, this process being repeated three times. The distilled water was next sucked off completely and the inclusion body allowed to break up. Then the coverslip was inverted over the moist chamber of the microdissection apparatus. To make an inoculation from the inclusion body smear a 4 mm. solid glass rod, which had been drawn to a fine point, was clamped in the Chambers apparatus and forced across the smear. A resulting faint scratch could usually be seen in the substance of the smear along with a minute accumulation of material on the glass point. The tip of this glass point, carrying its small portion of the smear, was carefully inserted into the defeathered follicle of a normal chick and then broken off, to be left, glass and all, in the follicle. As many as ten scratches across the smear of a single inclusion body have been made in this fashion, using a fresh, sterile, glass point for each scratch and inoculation. In each instance all of the scratch inoculations from a given inclusion were made into the follicles of a single chicken. After the completion of the inoculations the coverslip was removed from the moist chamber, stained and mounted and kept as a permanent record of the experiment (Fig. 5).

The experiment has been repeated with seventeen different inclusion body smears. In all, 135 inoculations have been made, fifty-two of which resulted in characteristic fowl-pox lesions. The largest

number of successful inoculations from a single smear was six, obtained in two instances, while from each of three smears only a single "take" was obtained.

The determination of a "take" was based upon the development of the characteristic firm, white swelling of the follicle (Fig. 7). In case any doubt existed, the follicle was removed and digested in trypsin, a positive result then being based upon the demonstration of inclusion bodies.

TABLE I

Showing Results of Scratch Inoculations from the Smears of Single Inclusion Bodies

Number of inoculated chicken	Number of scratch inoculations from the inclusion body smear	Number of positive results	Control inoculations (all negative)
WH 89	6	3	*No control*
WH 92	3	2	*No control*
WH 93	8	5	*No control*
WH 104	10	4	10
WH 105	10	2	10
WH 106	10	3	10
WH 107	10	1	10
WH 108	10	1	10
WH 109	10	2	10
WH 110	10	2	10
WH 111	10	4	10
WH 112	6	6	6
WH 113	6	2	6
WH 114	4	1	4
WH 115	8	6	8
WH 116	6	5	6
WH 117	8	3	8
Total	135	52	118

As indicated in the above table, no control inoculations were made in the first three experiments. In the fourteen subsequent experiments control inoculations were made by running sterile glass points through the pool of distilled water used in the final washing of the inclusion body. The points were run close to the body, but care was taken that they should not actually touch it. The tips of these glass points were then broken off in feather follicles on the opposite breast of the same chicken that was to be used for inoculations from the inclusion body smear (Fig. 7). All 118 of these control inoculations

were negative. Evidently any fluid from the final wash pool which might have adhered to the glass points was non-infectious. Furthermore, the control inoculations demonstrate that the breaking off of the glass points in chickens' follicles does not in itself produce a foreign body reaction in any way suggestive of the fowl-pox lesion.

Discussion

In the technique of these experiments too many variables are involved to permit a positive statement as to the reason for the great variation in the number of "takes" from a single inclusion body. A cursory examination of the table indicates that in general, the percentage of "takes" was much lower where ten inoculations were attempted from a single smear than where fewer inoculations were made. The element of fatigue on the part of the experimentalist may enter here, for the process of washing and inoculating is a tedious one and it is difficult to introduce one of the fine glass points into the opening of a feather follicle without accidentally brushing it against the outside of the follicle. This latter accident might well brush the infectious material from the point.

With the inoculations WH107 and WH108 a slightly different technique was employed. In "107" the glass points had been dipped in sterile gelatine preceding the scratching process, in the hope that the gelatin might cause the adherence of more infectious material to the point. In "108" hollow points drawn from glass tubing were used instead of points drawn from solid glass. On account of their ineffectiveness these technical variations were abandoned following a single trial of each, and the solid glass points were used in all the other inoculations.

A further factor which was apparently of some importance in the inoculations was the "age" of the inclusion bodies used. In general, better results were obtained with an inclusion from a seven- to ten-day fowl-pox lesion than with one from a lesion two weeks old or older.

The chief significance of the present series of experiments, lies, we believe, in the demonstration of the infectious nature of material from seventeen different inclusion bodies which had been digested and washed free of all particles of cellular material and then allowed to disintegrate so as to liberate their component Borrel bodies. Also of

importance is the demonstration of the capacity of such an inclusion body to furnish infectious material for as many as six positive inoculations. The limits of this subdivision of an inclusion body we feel has by no means been reached.

It is impossible to say from these experiments that one specific portion of the inclusion body smear alone was responsible for the successful inoculations. Obviously some of the ground substance of the body is picked up on the glass point as well as the Borrel bodies. However, the lipoid component of this ground substance has been demonstrated to be non-infectious by inoculation of both alcoholic and ether extracts of the inclusion bodies, and we have not as yet been able to isolate any protein component of the inclusions distinct and apart from the Borrel bodies. These facts, together with the enormous numbers of Borrel bodies in a single inclusion and their perfect uniformity in size and shape, lead us to think that in our various experiments the Borrel bodies represent the important part of the inoculum — the actual virus of fowl-pox.

Summary

1. Inclusion bodies of fowl-pox may be broken up by using the surface tension of a drying film of water. The stained smear of an inclusion body thus disrupted has been shown to contain as many as 20,000 Borrel bodies — minute coccoid structures uniform in size and shape.

2. Multiple inoculations have been made from the smears of isolated and ruptured inclusion bodies with as many as six inoculations from a single inclusion resulting successfully. Control inoculations were all negative.

3. The lipoid component of the inclusion bodies is non-infectious. Aside from this lipoid, the Borrel bodies form the major constituent of the inclusions and are judged to represent the actual virus of fowl-pox.

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DESCRIPTION OF PLATES

PLATE 129

FIG. 1. Inclusion bodies in saline. × 180.

FIG. 2. Same inclusion bodies thirty minutes after distilled water had been substituted for the saline. × 180.

FIG. 3. Inclusion body swollen in distilled water. × 1000.

FIG. 4. Smear of single inclusion body broken up by the surface tension of a drying film of water. Borrel bodies are seen as the minute coccoid structures. Morosow's stain. Photomicrograph taken with blue light. × 510.

FIG. 5. Inclusion body smear from which the six "scratch" inoculations of WH112 were made. The marks of two of the scratches are plainly visible. Morosow's stain. Photomicrograph taken with blue light. × 360.

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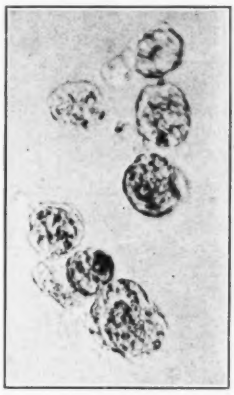

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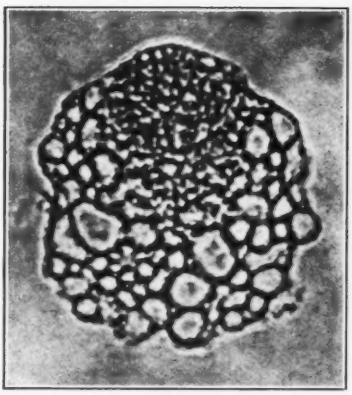

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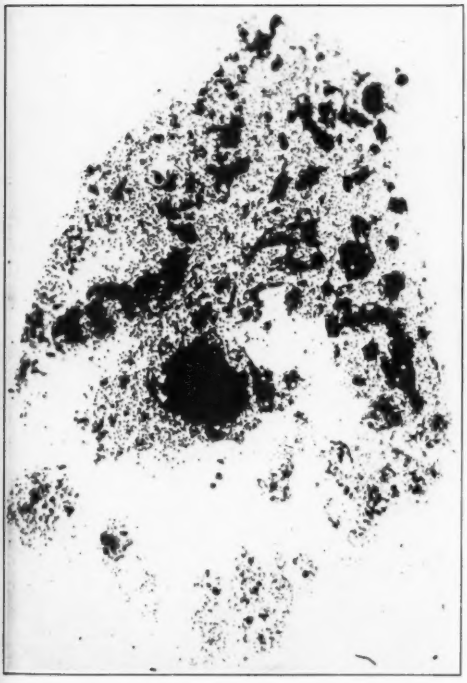

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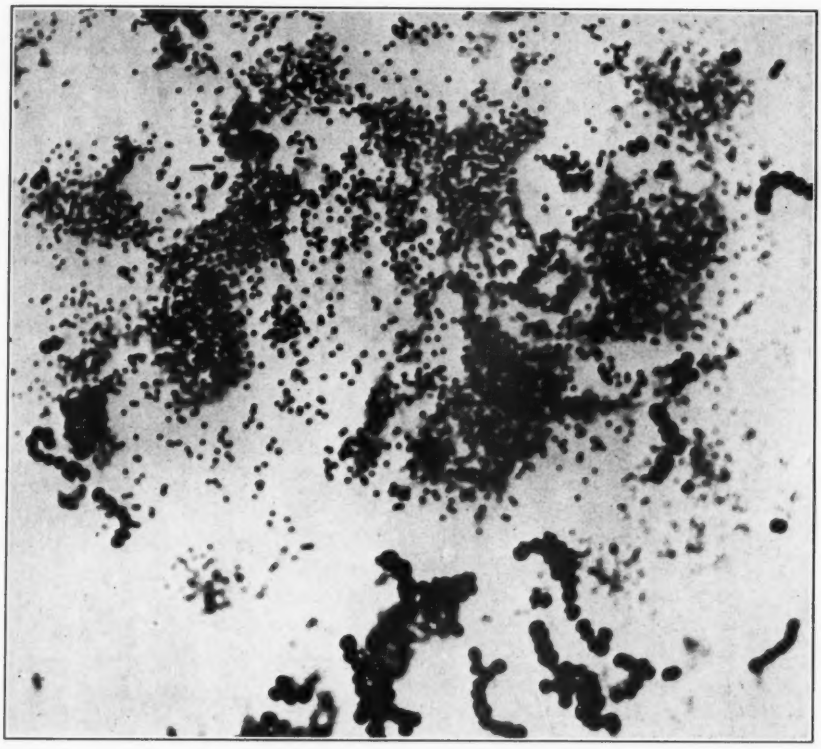
5

Woodruff and Goodpasture Specific Cellular Inclusions of Fowl-Pox

PLATE 130

FIG. 6. Smear showing comparative size of Borrel bodies and hemolytic streptococci. Morosow's stain. Photomicrograph taken with blue light. × 2200.

FIG. 7. Skin from breast of chicken showing at left three follicles ten days after each had been inoculated with material scratched up from a single inclusion body smear. Control inoculations at right. × 0.8



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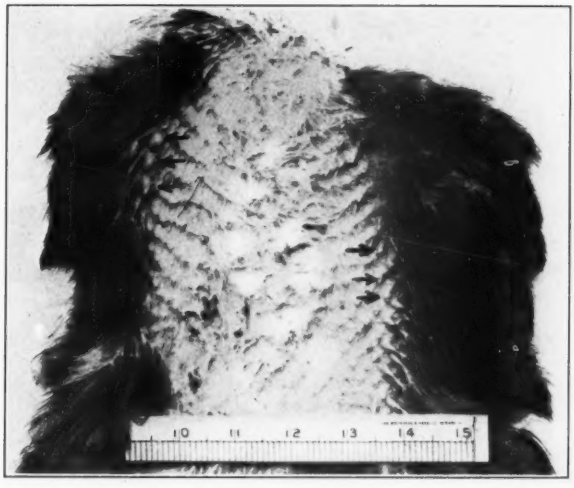

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RHINOSPORIDIUM SEEBERI: PATHOLOGICAL HISTOLOGY AND REPORT OF THE THIRD CASE FROM THE UNITED STATES *

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The organism now known as *Rhinosporidium seeberi* has been described from three geographical regions, far distant one from another, Argentina, India and the United States. The instance of infection with it which is reported here is believed to be but the third from this country. Likewise but three cases of this disease have been described from Argentina, as far as we can learn. In India, however, it has been recognized many times since its first description from that country in 1903. There are in the literature at least twenty-five case reports from India and Ceylon, several of them dealing with the occurrence of this infection in anatomical locations other than the nose.

The first description of this parasite was published in a thesis from Buenos Aires in 1900, by Guillermo Seeber.[1] We have been unable to obtain this in its original form but a quite complete abstract can be found in the historical section of Ashworth's [2] paper on Rhinosporidium. The patient was an agricultural laborer, 19 years of age. Although born in Italy, this young man had lived in Argentina for eighteen years. He presented an obstructing polypoid growth of the left nasal fossa projecting through the external orifice. Upon this material, and upon that obtained at a second operation necessitated by recurrence, Seeber's description of the organism was based. In an appendix, note is made of a still earlier, but unpublished case, observed in 1892 by Professor Malbran of Buenos Aires. In 1912 Seeber republished a summary of his thesis and at that time referred to still another case found by Malbran, thus bringing the total to three for Argentina. It was this republication which brought the unrecognized priority of Seeber to the attention of other investigators in this field.

In 1903 O'Kinealy [3] described as a local psorospermiosis a growth from the left nostril of a male Mohammedan, 22 years of age. This

* Read at the meeting of the American Association of Pathologists and Bacteriologists, New York City, April 17, 1930.
Received for publication August 9, 1930.

man was a native of Bihar and had been working as a mason for two and one-half years. However, he had first noticed the growth in his nose three years before while working in a hide store. He was seen by O'Kinealy in Calcutta in 1894. There had been two previous partial removals of an obstructing polypoid mass and when the patient was last seen there was again evidence of recurrence following a third operation. Upon examination he presented a small vascular pedunculated tumor about the size and shape of a large pea, freely movable, painless and having the appearance of a papilloma. It was attached by a short pedicle to the mucous membrane at the anterior and upper part of the cartilaginous septum, was friable and bled freely during the course of its removal. The pathological description was furnished by Major J. C. Vaughan. In this report O'Kinealy stated that he had reason to believe that similar cases had been observed in Calcutta, though he did not think that they had been published.

A few sections from the case described by O'Kinealy were reworked by Minchin and Fantham,[4] who, in 1905, gave a further description of the parasite, differing in some particulars from that of O'Kinealy. At this time they proposed the name *Rhinosporidium kinealyi* for the etiological agent.

In 1906 Beattie[5] described the material from a nasal polypus which had been supplied him by Dr. Nair of Madras, who stated that he had had four similar cases. All of these were from the state of Cochin on the west coast of India. This material was seen by Minchin and was pronounced similar in every respect to that from which he had described *Rhinosporidium kinealyi*. In one of his papers Beattie refers to the cysts produced as being 6 to 8 mm. in diameter. These measurements were obviously incorrect and Ashworth[6] has found no cysts exceeding 0.2 to 0.3 mm. in diameter in the very slides which Beattie used. In the following year Beattie[7] recorded the occurrence of the same organism in aural polypi, but without a detailed case report.

The first American case was described by J. Wright[8] in 1907. Wright had received this material from Dr. E. C. Ellett of Memphis, Tennessee, in 1903, but it was not diagnosed until after the appearance of the papers by O'Kinealy, Minchin and Fantham, and Beattie. The patient was a young farmer, 20 years old, who had never been away from the neighborhood of Memphis. He had sub-

mitted to a series of operations beginning with the removal of a growth from the lower anterior part of the right septum in 1897. The second operation, in 1898, was upon the anterior portion of the lower right turbinate. Still another operation was performed in March, 1902, and the removal of the specimen examined was accomplished by Ellett in December of the same year. He found three areas involved, the old sites on the septum and turbinate and a new area on the septum. There was no evidence of recurrence two years later.

Growths produced by this same organism were described from the conjunctiva by Ingram [9] in 1910, and by Elliot and Ingram,[10] and by Kirkpatrick [11] in 1912, a total of three cases. The anatomical distribution of the lesion resulting from this infection was extended further by the description by Ingram in his earlier papers of a growth of six years duration involving the entire glans penis of a patient 45 years old.

In 1912 Seeber published a summary of his earlier thesis which was thus brought to the general knowledge of protozoölogists. His priority was established by investigations instituted by Ashworth [2] for it was found that the name *Coccidium seeberi* had been assigned to this organism in Belou's *Parasitologia Animal* in 1903. Accordingly Ashworth emended the name to *Rhinosporidium seeberi*, which has been generally accepted since his monograph appeared in 1922.

Tirumurti [12] collected, in 1914, fifteen instances of Rhinosporidium infection in each of which tissue specimens had been examined in the pathological laboratory of the Madras Medical College. Several of these had been reported previously by other authors, but in addition to these, he described eleven new cases of nasal and nasopharyngeal involvement. Further case reports have followed. From the General Hospital of Colombo, Ceylon, Chelliah,[13] in 1918, described three additional examples from the nose in males, aged 17, 19 and 58 years respectively. Two of his patients had never been in India and one had never been out of Ceylon. Two years before Kirkpatrick[14] had reported for the first time the occurrence of Rhinosporidium infection in the lacrymal sac. In 1922 R. E. Wright [15] recorded three conjunctival cases which had been seen within a period of three months in the Civil Orphan Asylum in Madras. The patients were Eurasian boys whose ages were 13, 14 and 14 years of age. One of these had a growth on both the upper and the lower palpebral

conjunctiva. In a second paper [16] the same author described the treatment and apparent cure of one of his conjunctival cases by the use of 2 per cent tartar emetic dropped into the eye three times a day over a period of three months, and also recorded a new case in which there were large polypi upon the inferior and middle turbinates. In this patient the lacrymal sac, the size of a filbert, was found upon extirpation to consist of a fibrous wall with polypoid projections completely filling the interior. In a subsequent report, with Tirumurti, Wright [17] referred to the occurrence of the same parasite in a papillomatous growth of the uvula.

The entire field of information concerning Rhinosporidium was very completely and critically reworked by Ashworth [2] in 1922. By him the designation *Rhinosporidium seeberi* was established as previously described and an extremely thorough and systematic morphological description made. He corrected numerous mistakes in the earlier accounts and worked out, on the basis of morphology alone, an acceptable life history. We find the organism in our own case to be in every significant respect in accord with Ashworth's description.

The second North American case was described by Drs. Mary C. Lincoln and Stella M. Gardner [18] in 1929. The patient was a man, 40 years of age, who was born near Carthage, Illinois, and who had lived there until he was about 17 years old. He then spent three years in Chicago and one year in Oklahoma. In 1925 he was in Florida for nine months. He had never been outside of the United States. Thirty years before he had had an operation performed on his nose and at that time the septum was perforated. There was no further difficulty for twenty-two years until he commenced to have a discharge and occasional bleeding. Eight years later the polypoid tumor from which the diagnosis was made was removed by Dr. M. C. Van de Venter of Keokuk, Iowa. The specimen had the appearance, grossly, of an ordinary nasal polypus. Microscopic sections showed the organisms in large numbers and in practically all stages. Very good photomicrographs are used to illustrate the more important morphological features of the organism. These leave no doubt that the parasite has the same structural characteristics as did those described by Seeber from South America and by the various workers upon the material coming from India and from Ceylon.

History of Case (A.D.R.)

The patient was a male engineering student, aged 26 years, who was born at Clarksdale, Missouri, and who had never been outside of the United States except for three days spent in Canada. He had had no close association with any foreign-born person. He had, however, lived on a farm for twelve years, from the age of 6 to 18 years. During this period he was undoubtedly playing and working in close contact with horses and cattle.

His mother and father were American-born and they, as well as five brothers and two sisters, were living and in good health. No other member of the family had experienced a similar condition.

The patient had first noticed the lesion in his nose in February, 1926, following an injury received while wrestling during the preceding month. At that time the nose was tender and painful to the touch but no history of unusual discharge, foul odor, or bleeding was obtained. Shortly thereafter the right side of the nose was operated upon for nasal polypi by Dr. J. M. Brown of Maysville, Missouri. No further trouble was noted until after a second injury while boxing in February, 1928. Progressive obstruction of the right side of the nose developed and had become almost complete at the time of a second operation in May, 1929.

When the patient presented himself for examination on June 22, 1929, there was found a polypoid growth of the nasal mucosa attached to the right septal wall about 1.5 cm. within the vestibule of the naris. There was a marked induration of the base and area of attachment and some granulation tissue formation. The greater part of the polyp was a grayish, semitranslucent rounded mass with a slight amount of denser vascular tissue extending into it at the base. The polypoid structure was removed with the snare and the base cauterized. Recovery was uneventful and there was no indication of recurrence four months after removal.

Pathological Description

The specimen when received was a small rounded mass looking not unlike an ordinary nasal polypus. It was bisected before being impregnated in paraffin and revealed no feature attracting attention. It was not, however, examined closely as would have been done had its true nature been suspected.

The Indian physicians believe that the polypoid rhinosporidial growths can be recognized as such by naked-eye examination. The smaller polypi are said to be very similar to a raspberry (Tirumurti [12]) in form and color. These growths are friable, tearing easily and bleeding severely when torn. Various writers have described a central, branching, vein-like supporting stroma. The larger parasites within the tissue are visible to the unaided eye as white spots of pin-point size.

Our material was examined first as stained routinely with hemalum and eosin. Later, additional sections were stained with Heiden-

hain's iron hematoxylin and eosin, Unna's methylene blue, Löffler's methylene blue, Gram's stain, carbol fuchsin and methylene blue, Giemsa's stain and phenol iodine green. Routine hemalum and eosin, and eosin-methylene blue staining were found to be sufficient for diagnostic purposes and for examination of the morphology of the infecting organism. The special stains were of value in securing photographs of certain structural details.

Upon examination with the lower powers of the microscope the specimen showed the structure of an inflammatory polypus with a moderately vascular, edematous, connective tissue stroma. In the stroma there were many infiltrating cells, chiefly lymphocytes and plasma cells but with a few polynuclear and polymorphonuclear leucocytes. As compared to the usual type of edematous nasal polypus occurring in chronic hypertrophic rhinitis, the stroma was denser and showed more fibroblastic proliferation, less edema and but very few eosinophiles. Nowhere was the cellular infiltration distinctly purulent. The most striking feature was the presence of the characteristic parasitic cysts, each a single organism, in various stages of development. Averaging about 100 microns in diameter, these cysts were closely placed throughout the polypus so that more than one-half the area in any field was covered by them (see Fig. 1). Each cyst was surrounded by a doubly-contoured chitinous-appearing capsule which showed concentric lamination when favorably stained, and especially in the more mature individuals. The younger organisms, when cut through the central part, showed a well stained karyosome about which there were aggregated nutrient granules and vacuoles containing materials staining in part with basic, and in part with acid, stains (see Figs. 2 and 3). Some of the cysts showed irregularity of form apparently resulting from mutual pressure. By some of the earlier authors this deviation from spherical form was thought to indicate ameboid movement.

Accepting the developmental life history of *Rhinosporidium seeberi* as worked out by Ashworth, practically all stages can be illustrated from our material. There is no evidence presented which is contradictory to the results of his investigation. The early development of the trophic stage can be illustrated by a parasite measuring but 8 microns in diameter, but already showing the doubly-contoured wall (Fig. 4). No trophic granules are present. By the time the parasite reaches a cyst-size of 60 microns the trophic granules

are abundant and the nucleus undergoes changes preparatory to its first division (Fig. 5). Shortly thereafter the chromatin is partially extruded from the karyosome in the form of coarse threads (Fig. 6). With further nuclear divisions the parasite increases in size and the envelope is thickened by the addition of concentric laminae on its inner surface so that it now becomes very distinctly striated. This is especially marked about one region where an annular thickening marks the site of the future pore for the discharge of the mature spores. The nuclei have now considerably increased in number and are scattered through the cytoplasm in which there is still abundant trophic material. In a later stage the nuclei multiply until they pack the interior of the cyst and the cytoplasm becomes concentrated about them and apportioned to them, completing cytoplasmic division (Figs. 6 and 7). With the maturation of a portion of the spores the parasite, which may now be termed a sporangium, reaches a size of 200 to 300 microns. The pore is now ruptured and the spores escape (Fig. 10). Such of the spores as are mature develop a limited number of spherical granules, 4 to 16, which are apparently distinct from the centrally placed karyosome and which according to recent investigations are not reproductive bodies (Fig. 11). With the escape of the mature spores the cycle is complete as far as is now known. It seems probable that such spores can proceed to the development of the early trophic stage directly, although full evidence in respect to this stage is lacking.

The emptying and collapse of the ripe sporangium is followed by a local proliferative reaction of foreign body type. The remains of the wall and such immature spores as are present may be included within multinucleated giant cells which persist for some time, giving the usual type of foreign body pseudotubercle formation. Foreign body giant cells have not been observed in the granulation tissue of this infection except in connection with the disposition of the remains of ruptured sporangia as here described.

General Considerations

Systematic Position of the Organism: As a result of reworking the morphology and probable life history of *Rhinosporidium seeberi*, Ashworth found it necessary to assign it an entirely new systematic position. He believes it to belong to the lower fungi and not to the sporozoa, and tentatively places it in the suborder Chytridineae.

Geographical Distribution: Since there seems to be morphological identity on the part of the causal organism in all cases described as due to *Rhinosporidium kinealyi* or *Rhinosporidium seeberi* we must conclude for the present, at least, that but one species is concerned with the production of the disease in question. The geographical distribution then becomes all the more remarkable for its apparent discontinuous character. Most of the cases have been found in southern and western India and Ceylon. Then we have the early group of three cases from Argentina and finally the three scattered cases from the United States. The first of these patients had never been far from Memphis, Tennessee; the second had spent most of his life in Illinois, but had been in Florida and Oklahoma; our present patient was born in Missouri and had spent the greater part of his childhood on a farm, but was in Michigan at the time the polypus was removed. In view of such a wide but discontinuous distribution, it seems probable that infection with *Rhinosporidium seeberi* is much more common than the few reported cases would seem to indicate. Clinically, the condition could not possibly be recognized except by those thoroughly familiar with it. Only microscopic examination of the tissue removed can bring about its recognition. Can it be that many cases considered ordinary polypi and not examined microscopically are due to this infection? Our own experience seems to discredit that explanation. We have routinely examined all nasal polypi removed at the University Hospital as well as many others sent to this laboratory from other sources. Although the total reaches many hundreds no other example of this infection has been found. The peculiar geographical distribution could be readily explained if it were found that some lower animal is a frequent carrier of this organism and man but an occasional recipient. From case reports it is evident that many of those suffering from this infection have lived on farms or have been in close contact with farm animals or the products of farm animals, such as hides. It is not at all certain that this is true of all. It is of great interest that a similar condition has been described for the horse in South Africa, the causal organism having been named *Rhinosporidium equi* by Zschokke.[19]

Age and Sex Incidence: Rhinosporidium seeberi infections occur particularly in young men, although no age is exempt. It has been found in boys as young as 10 years and in men 60 years of age. It is remarkable that not one of the forty or more reported cases has been

in a female. It must be that the male is exposed to the infection in some manner which is not shared by the other sex. Wright [17] found three cases involving the conjunctiva in boys in the Civil Orphan Asylum in Madras. Not one of the girl inmates living under precisely the same conditions was affected. Both groups used the same artificial swimming pool, in which the water was changed once in two or three weeks, and the same dusty playground.

Anatomical Distribution: While the earlier examples of infection with *Rhinosporidium seeberi* were all of the nasal and nasopharyngeal area, subsequently described cases have extended the anatomical distribution to include the aural canal, the conjunctiva and lachrymal sac, the uvula and the penis. Tirumurti [12] predicted its eventual recognition from the external auditory meatus, mucous membrane of lips, cheek and tongue, larynx, vagina and rectum. Since in the nasal tract infection occurs in areas covered by either columnar or squamous epithelium, there seems to be no good reason why this prediction will not be realized. With knowledge of the wider distribution, the designation *Rhinosporidium* has become much less appropriate.

Mode of Infection: Nothing is known with certainty about the mode of infection and of transmission. The possibility of an animal host, probably among the larger farm animals, has already been mentioned. All efforts at experimental animal inoculation have given but negative results. Monkeys, guinea pigs and rabbits were tried by Ingram,[9] guinea pigs again by Tirumurti,[12] monkeys by Wright [16] and Cunningham, and mice, rabbits and guinea pigs by Rettie, working with the material from Ashworth's case.[2] Likewise, with one possible but unproved exception, all attempts to grow this organism have been unsuccessful. The possibility of contact infection is suggested in the histories of several patients who had been associated with others having a similar condition. A majority of the reported cases give no evidence of association with other cases. Finger-borne infection cannot explain those examples occurring well back in the nasal tract, the nasopharynx and the uvula. If the organism is transmitted in dust or water, an assumption in accord with its anatomical distribution, it is difficult to understand its limitation to males, as Ashworth points out. The occurrence of auto-inoculation is clearly shown by the development of additional polypoid growths in nearby but new sites.

Clinical Characteristics: Polypoid or papillomatous inflammatory newgrowth is the general characteristic of this condition. In the nasal tract this growth tends to become obstructive. Clinically it exhibits a marked tendency to bleed. After the usual form of operative intervention it practically always recurs, so that most patients give a history of repeated operations before the true nature of the process is recognized. There is no evidence of a generalized hematogenous dissemination in the reported cases.

Treatment: Wright was successful in the treatment of his case of Rhinosporidium infection of the conjunctiva with 2 per cent tartar emetic dropped into the eye three times a day. He therefore suggested similar applications for the nasal tract, possibly in the form of a daily spray and a weekly pack of the involved nostril. Usually the method of choice will be operation, but this should include the entire area of origin and not simply removal of the polypoid mass with snare or scissors. Patients should be advised of the likelihood of recurrence and of the desirability of frequent re-examinations.

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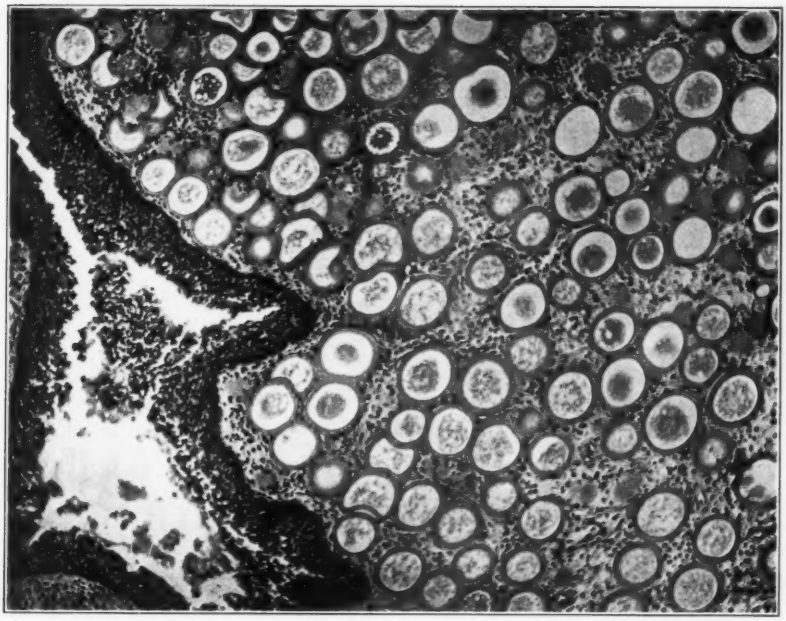
DESCRIPTION OF PLATES

PLATE 131

FIG. 1. Rhinosporidial nasal polypus. The closely approximated parasitic cysts fill the field, leaving but little inflammatory stroma visible. × 90.

FIG. 2. Higher power view showing the cellular stroma between the parasites. The organism in the middle of the field is cut so as to show the central karyosome and the surrounding nutrient substance. × 370.



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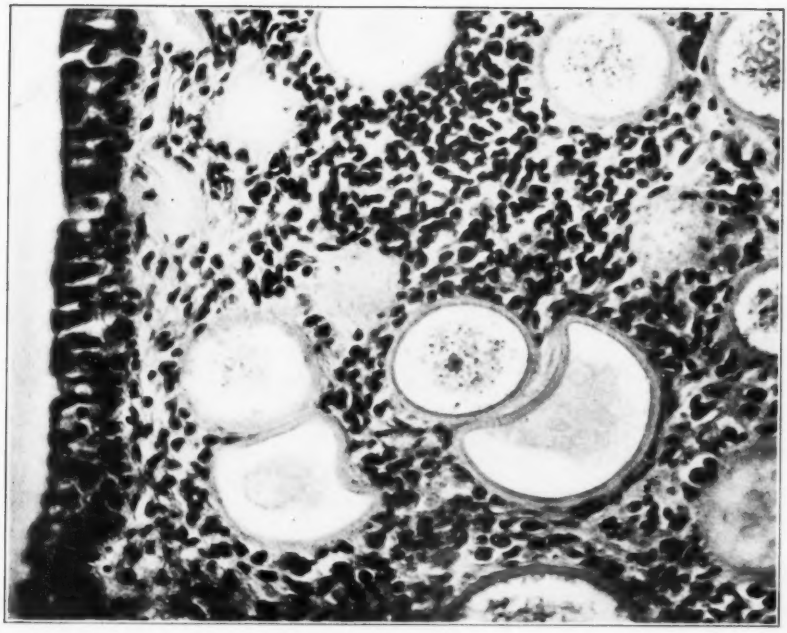

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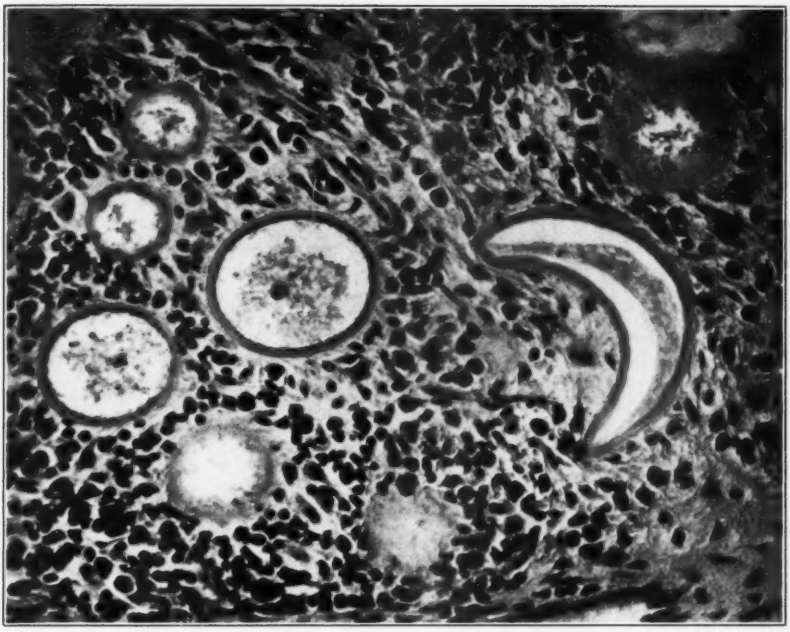
Weller and Riker Rhinosporidium Seeberi

PLATE 132

FIG. 3. Cellular stroma with fibroblastic proliferation. Two parasites show the central karyosome in the midst of the granular nutrient substance. × 370.

FIG. 4. Very early trophic stage of *Rhinosporidium seeberi*. Cyst 8 microns in diameter. The two-lobed arrangement of the chromatin is not constant. × 1500.



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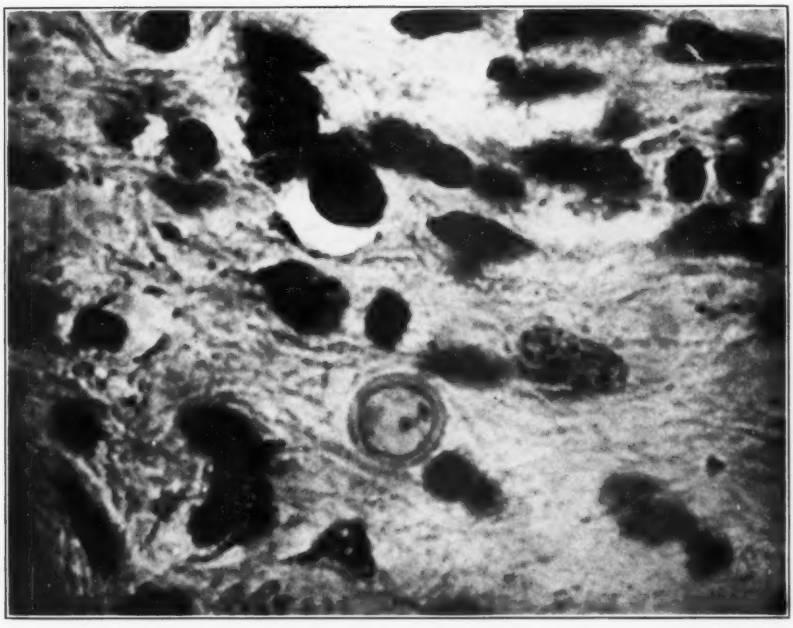

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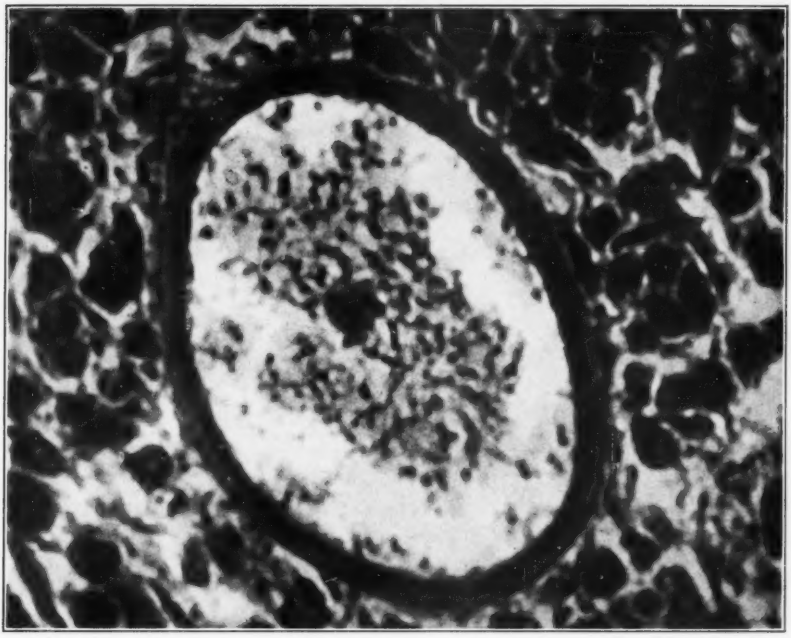
Weller and Riker Rhinosporidium Seeberi

PLATE 133

FIG. 5. Trophic stage 58 microns in greater diameter. Beginning segregation of chromatin preparatory to first nuclear division. Abundant trophic material. × 1500.

FIG. 6. Trophic stage 75 microns in its greater diameter. Early stage of first nuclear division. × 1500.



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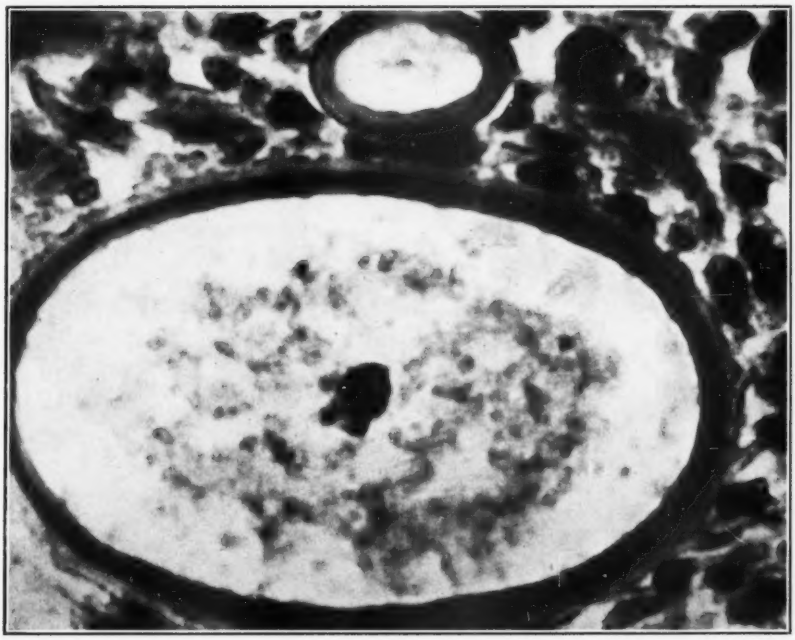


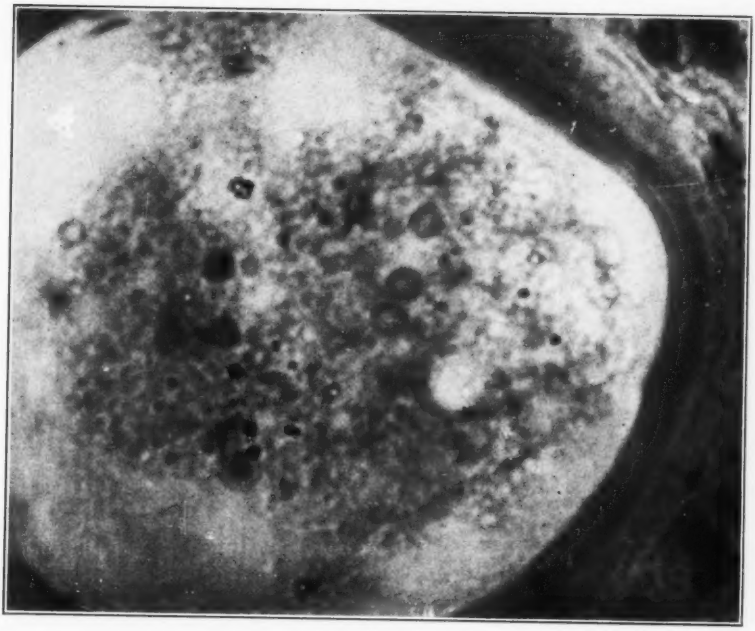
6

Weller and Riker Rhinosporidium Seeberi

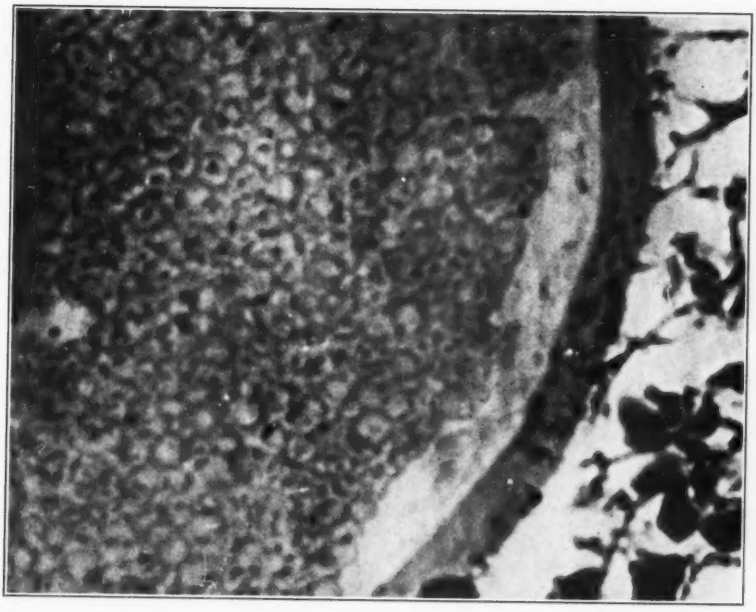
PLATE 134

FIG. 7. A portion of a parasite about 150 microns in diameter with thickened laminated wall and multiple nuclei. Twenty-eight nuclei were visible in the one section of this organism. × 1500.

FIG. 8. A portion of a parasite 210 microns in diameter, closely packed with maturing spores. Cytoplasmic division has now taken place. × 1500.



7



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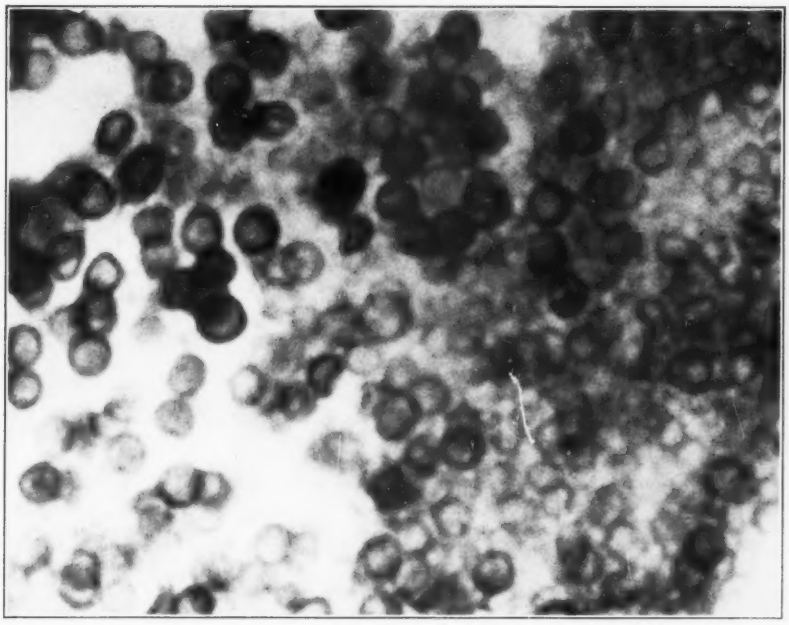
Weller and Riker Rhinosporidium Seeberi

Plate 135

Fig 9. Immature spores about 5 microns in diameter from the interior of a parasite in a somewhat later stage than in the preceding figure. × 1500.

Fig. 10. The escape of ripe spores through the pore. The thickened marginal annulus is well shown. About × 400.



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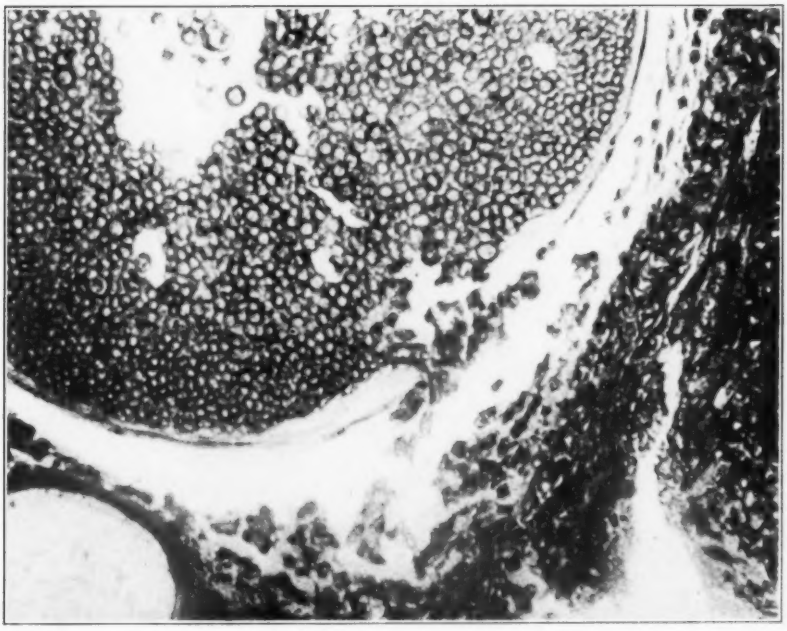


10

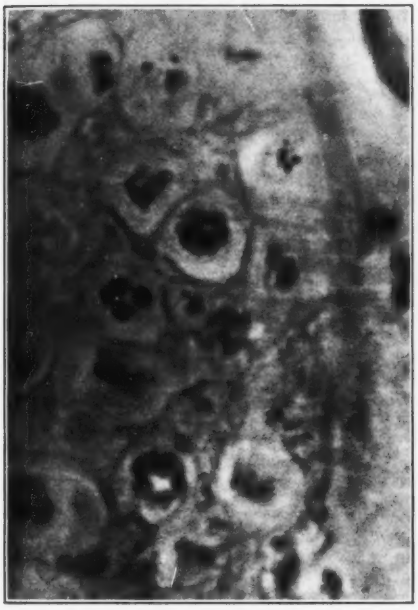
Weller and Riker Rhinosporidium Seeberi

Plate 136

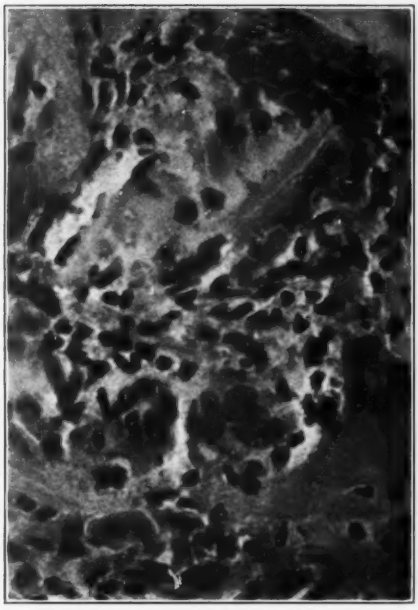
Fig. 11. Practically mature spores showing centrally grouped spherules with structureless material about them and well defined capsular membranes. About × 2000.

Fig. 12. Foreign body giant cell reaction about old sporangium. Portions of wall and spores included within multinucleate foreign body giant cells. × 300.

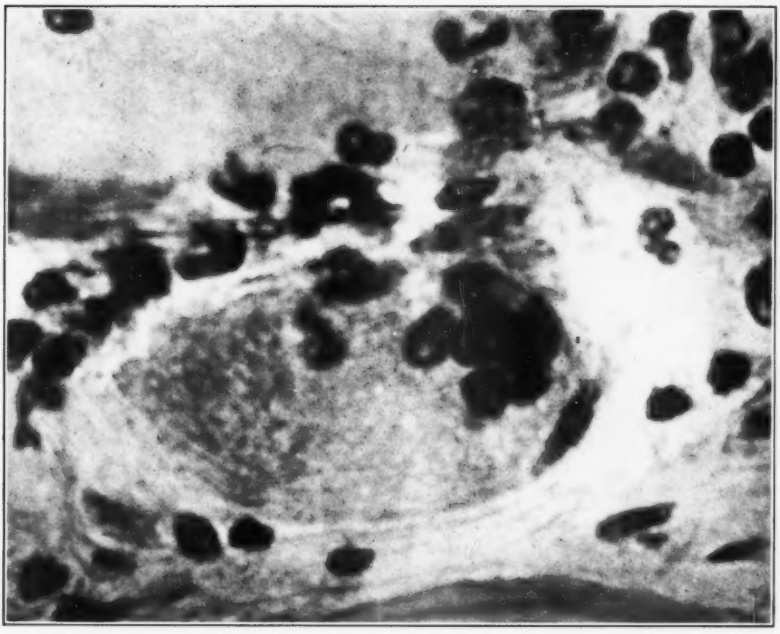
Fig. 13. Multinucleate foreign body giant cell containing many immature spores. Portion of wall of ruptured sporangium at bottom of field. × 500.



11



12



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Weller and Riker Rhinosporidium Seeberi

ENDOCARDIAL POCKETS *

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INTRODUCTION

In cases of insufficiency of the aortic valve, a coincidental and striking finding is that of endocardial pockets imitating the form of aortic valve leaflets on the surface of the interventricular septum of the left ventricle. These pockets are often multiple, their openings directed toward the aorta. Even more rarely such pockets are observed with their openings directed toward the apex of the heart.

LITERATURE

Zahn,[1] who first directed attention to these formations in aortic insufficiency, interpreted them as anatomical signs of incompetence of the aortic valves. He believed that such pockets occurred on the basis of simple endocardial thickenings which were brought about by the chronic irritation of the impulse of the regurgitating blood. In his opinion, the prolonged irritation of the regurgitating blood produced the pockets or pseudovalves only secondarily. He observed plain endocardial thickening in the left auricle in cases of insufficiency of the mitral valve, but he did not describe pockets in the left auricle. Herxheimer,[2] Dewitzky,[3] Rosenbusch,[4] Wilke,[5] and Cohn,[6] similarly believe in the mechanical genesis of circumscribed endocardial thickenings and endocardial pockets. Kaewel,[7] who states that the formation of endocardial pockets may aid in the diagnosis of aortic insufficiency, also traces back their origin in most of the cases to the mechanical irritation of the regurgitating blood. He thinks that continuous pressure of a thickened aortic leaflet of the mitral valve upon the opposite side of the interventricular endocardium of the left ventricle often cannot be ruled out as a contributory cause. Ziegler,[8] Aschoff,[9] and later Böger [10] emphasize that circumscribed endocardial thickenings are primarily inflammatory in nature, end

* Received for publication June 20, 1930.

stages of acute mural endocarditis. The pockets are formed secondarily, following the mechanical excavation of the thickened areas, by the regurgitating blood impulse after an insufficiency of the aortic valve has been established. Krasso [11] maintains that primarily all types of pockets are formed on the basis of endocardial thickenings which are caused mechanically by the force of the regurgitating blood stream, but infectious thrombi with organization, formed in such areas, might also play a rôle in the formation of circumscribed endocardial thickenings.

Schmincke [12] and Borst [13] are of the opinion that the formation of endocardial pockets is a sign of functional adaptation.

Sotti [14] holds that most of the pockets are abnormal muscle bridges converted into connective tissue, or aberrant muscles, or chordae tendineae, and therefore should be classified rather as malformations of the heart.

Ribbert,[15] in Henke and Lubarsch's handbook, says that several causes might lead to pocket formation; a primary circumscribed endocarditis, a continuous friction of thickened or calcified aortic leaflets of the mitral valve upon the opposite endocardial surface of the interventricular septum, the mechanical irritation of the regurgitating blood in aortic insufficiency, and finally, congenital anomalies.

The foregoing short survey of the literature shows the different views expressed as to the origin of endocardial pockets. While many writers believe they occur on a purely mechanical basis, others believe them to be of inflammatory origin, while the opinion is expressed by some authors that they are formed on the basis of congenital malformation. A possible relation of the endocardial pockets to the type of valvular disease has not attracted attention.

The present study was undertaken, first, to determine whether or not by the use of serial sections one definite etiological factor might be demonstrated as to the cause of the formation of pockets; secondly, to see if in different types of valvular diseases a different cause could be found to explain such formations. Endocardial pockets of the left auricle which were present in one case were similarly studied.

Methods

Six hearts were studied. A detailed description of the hearts will be given subsequently. The hearts were obtained from routine post-

mortem examinations. No museum specimens were used for this investigation. The endocardial pockets with the adjacent myocardium were cut out, hardened in 10 per cent formalin and embedded in paraffin. Serial sections were made from the various pockets. The first, fourth, seventh, etc., sections were stained with iron hematoxylin and eosin; the second, fifth, eighth, etc., according to Van Gieson's method, while the third, sixth, ninth, etc., were prepared with a combination of iron hematoxylin and orcein stain, using a method described elsewhere (Saphir [16]).

Case Reports

Endocardial Pockets in Hearts Showing Acute Ulcerative and Chronic Endocarditis (Subacute Bacterial Endocarditis with Positive Blood Culture of Streptococcus Viridans)

Case 1. *Pathological Diagnoses:* Acute ulcerative and vegetative endocarditis superimposed on chronic endocarditis of the aortic and mitral valves, with insufficiency of both valves.

Gross Description: The heart was enlarged, weighing 450 gm. The free margins of the cusps of the aortic valve showed several fresh vegetations. The cusps themselves were retracted and shortened. There were a few small ulcers throughout the ventricular surface of the left aortic cusp. Many recent vegetations were found extending over the aortic leaflet of the mitral valve. The free margins of the mitral valve leaflets were retracted and thickened, the chordae tendineae were firm and shortened. The endocardium below the left aortic cusp showed many recent, grayish red vegetations. The endocardium surrounding them was of a grayish white color. At a distance of 6 mm. from these vegetations, in an area 2 cm. below the aortic valve, situated on the interventricular surface of the left ventricle there were two small circumscribed endocardial pockets measuring 3 and 5 mm. in width. These pockets were open toward the aortic valve.

Case 2. *Pathological Diagnoses:* Acute vegetative, superimposed on chronic endocarditis of the aortic valve. Acute vegetative and ulcerative endocarditis of the mitral valve superimposed on chronic endocarditis. Insufficiency of the aortic and mitral valves.

Gross Description: The heart was enlarged, weighing 500 gm. The free margins of the aortic cusps were thickened, the cusps re-

tracted and to some extent adherent to one another. A few small, recent vegetations were found attached to the free margins. The mitral leaflets were markedly thickened, in part calcified, and covered with large, partly firm and partly friable vegetations. Throughout the aortic leaflet of the mitral valve on its ventricular aspect, several ulcers were noticeable. The endocardial surface showed throughout the interventricular septum of the left ventricle, beginning from an area of about 3 cm. below the aortic valve and extending down to an area of about 2 cm. from the apical portion, a number of pockets. These pockets varied in size from 4 to 12 mm. in width. Some of the pockets extended in the form of fibrous bands over several trabeculae carneae and reached the papillary muscles. All pockets were open in the direction of the aortic valve. While some pockets were found in the vicinity of vegetations of the aortic leaflet of the mitral valve, others showed no apparent relation to these vegetations. There were no thrombi close to the pockets. The cusps of the pockets were grayish in color, firm in consistency, and free from vegetations.

Histological Examination: The histological findings in the pockets of both cases were about identical. The endocardium in some of the sections was fibrosed and showed only a moderate number of nuclei. Many sections, however, showed a diffuse infiltration of lymphocytes, endothelial cells, and a few polymorphonuclear leukocytes and eosinophiles. The cells were apparently more abundant close to the point of attachment of the cusp of the pockets to the endocardium. Some slides showed the extension of the inflammatory cells into the subendocardial layer. The heart muscle fibers found in the subendocardial region were markedly atrophic, their cytoplasm distinctly granular in appearance. There were only a few inflammatory cells present in these portions. The cusps of the pockets showed in some of the sections a dense fibrous tissue with only a few cellular elements, but some of the sections again showed large numbers of endothelial cells, lymphocytes and a few polymorphonuclear leukocytes. A few fields showed the formation of small-sized blood vessels extending from the endocardium into the cusps. There were remnants of blood pigment present. In some of the sections remnants of organizing thrombi were recognizable. Sections which were taken from the bands extending over the trabeculae carneae showed the latter to be surrounded by connective tissue and elastic fibers with

many lymphocytes and endothelial cells. The trabeculae carneae themselves were outlined indistinctly and many of them could be recognized only with the aid of the Van Gieson stain. Between the fibers, inflammatory cells were observed. The elastic stain showed an abundance of elastic lamellae of the cusps. They were found in parallel rows. Some of the fields showed the extension of the endocardial elastic lamellae into the cusps.

Endocardial Pockets in Hearts Showing Syphilitic Involvement of the Aortic Valve

CASE 3. *Pathological Diagnoses:* Syphilitic aortitis with involvement of the aortic valve. Insufficiency of the aortic valve.

Gross Description: The heart was hypertrophic and dilated, weighing 650 gm. The aorta showed characteristic lesions of syphilitic aortitis. The cusps of the aortic valve showed a separation of the commissures extending over areas measuring 3 to 4 mm. in extent. There was a marked insufficiency of the aortic valve. The aortic leaflet of the mitral valve was free from pathological changes. The upper portion of the left ventricle, just below the aortic valve, seemed much narrowed as compared with the size of the remainder of the ventricle which was markedly dilated and hypertrophic. The impression was gained that this area which Krasso calls "conus arteriosus sinister," was the seat of a relative bottle-neck stenosis. The endocardium of the interventricular septum of the left ventricle showed a series of pockets in four parallel horizontal rows. The pockets measured 2 to 6 mm. in width. In the region of the trabeculae carneae a few connective tissue bridges were seen. All these pockets were open toward the aorta. Just below the left aortic cusp, however, another pocket was found which measured 3 mm. in width. This pocket was open toward the apex of the heart. In the vicinity of this pocket, the endocardium was grayish white in color and thickened.

CASE 4. *Pathological Diagnoses:* Syphilitic aortitis with involvement of the aortic valve. Insufficiency of the aortic valve.

Gross Description: The heart weighed 700 gm. It was markedly hypertrophic and dilated. The aortic lesions were characteristic of syphilitic aortitis. There was a distinct separation of the commissures of all three cusps and insufficiency of the aortic valve. The

mitral valve leaflets showed no changes. The conus arteriosus sinister (Krasso) was apparently the seat of a relative stenosis. The endocardium of the left ventricular surface of the interventricular septum showed many thickened areas which were of stringy appearance and which were found in an area about 2 cm. below the aortic valve. In and below this region several typical pockets were found which measured from 2 to 8 mm. in width. The cusps of these pockets were firm in consistency. Most of the pockets were in one horizontal row, but several were found above and below this row. All these pockets were open toward the aortic orifice. Just below the left aortic cusp there was one pocket which measured 5 mm. in width, and which was open toward the apex. The endocardium close to this pocket was grayish white in color and of much firmer consistency than the surrounding portions.

Histological Examination: The endocardium in the region of the pockets was fibrosed. There was an occasional lymphocyte and endothelial cell found, but the tissue in general was very poor in cells. In some portions the endocardium was hyalinized. The fibrosis extended into the surrounding subendocardial layer. But here, too, very few cellular elements were seen. The muscle fibers close to the endocardium were atrophic and in some fields present in the form of a light pink-stained material assuming the shape of heart muscle fibers. By use of the Van Gieson method these fibers stained yellow. The cusps of the pockets showed a dense connective tissue overgrowth with a varying number of fibroblasts. There were hardly any blood vessels found in this portion nor was there any evidence of organization. Both types of pockets, the ones with the openings directed toward the apex and the ones with the mouths open toward the aorta, showed identical lesions. The elastic tissue stain showed an abundance of elastic fibers throughout the cusps. Some of the sections showed clearly the extension of the elastic lamellae from the endocardium into the cusps.

Endocardial Pockets in Hearts Showing Rheumatic Endocarditis

CASE 5. *Pathological Diagnoses:* Healed endocarditis of the aortic valve. Stenosis of the aortic orifice.

Gross Description: The heart was enlarged and dilated, weighing 400 gm. The free margins of the aortic valve were thickened and

showed adhesions between the lateral portions of the cusps, producing a stenosis of the aortic orifice. The myocardium histologically showed several Aschoff bodies. The endocardium in an area about 1 cm. below the left aortic cusp showed a pocket measuring 8 mm. in width. This pocket was open toward the apex. The margin of this pocket was thin and sharp. The endocardium in the neighborhood of this pocket was thickened and fibrosed.

Histological Examination: The sections of the endocardium showed a marked increase of connective tissue with only very few nuclear elements. The fibrosis extended into the surrounding portions of the myocardium. The heart muscle fibers in this region were apparently atrophic. The cusps themselves showed a hyalinized connective tissue with few spindle-shaped cells. There were no blood vessels found, nor was there any other evidence of organization.

CASE 6. *Pathological Diagnoses:* Acute verrucous, superimposed on chronic endocarditis of the aortic and mitral valves. Insufficiency of both valves.

Gross Description: The heart was enlarged and dilated weighing 300 gm., (patient was a child 6 years of age). The cusps of the aortic valve were shortened and retracted. Their free margins were studded with a row of bead-like vegetations. The free margins of the mitral valve were thickened and retracted. Some of the chordae tendineae were fused by confluence. They were much shorter than normal, and thickened. The myocardium upon histological examination showed many Aschoff bodies. In an area about 2 cm. above the mitral valve, the auricular endocardium showed two pockets. The pockets measured 3 and 5 mm. in width. They were quite separate and were open toward the mitral valve. The surrounding portions of the endocardium were grayish white in color and thickened.

Histological Examination: The histological examination of the endocardium in the region of the pockets showed a diffuse infiltration of many polymorphonuclear leukocytes, a few lymphocytes and endothelial cells. There was a moderate amount of connective tissue with many spindle-shaped cells. The cusps similarly contained a large number of polymorphonuclear leukocytes, many lymphocytes, endothelial cells, and, in addition, many connective tissue fibers with a large number of fibroblasts. There were small-sized blood vessels found extending into the cusps. Some of the fields showed

remnants of blood pigment and phagocytic cells. The elastic tissue stain showed a great number of elastic fibers without any particular arrangement. Some of the sections showed these fibers extending from the endocardium into the cusps.

Discussion

Endocardial Pockets in Hearts Showing Subacute Bacterial Endocarditis

The endocardial pockets which were found in the two cases of subacute bacterial endocarditis showed evidence of inflammation. There were still remnants of inflammatory cells, mainly lymphocytes, a few eosinophiles, endothelial cells and occasional polymorphonuclear leukocytes. Besides, a new formation of small-sized blood vessels was easily noticeable. Young connective tissue fibers were seen throughout some of the sections, while other sections showed scar tissue which in some portions was hyalinized. The sections emphasized the importance of serial sections as the only means of studying these changes. It easily can be understood why, by the use of only a few sections, hyalinized scar tissue alone might have been found. The sections showed that there must have been primarily an acute inflammatory exudate which secondarily became organized. Whether this was primarily a mural endocarditis or whether the initial lesion was brought about by contact with the diseased aortic leaflet of the mitral valve cannot be decided.

Both of our cases showed an insufficiency of the aortic valve. It seems plausible that the force of the regurgitating blood and pressure during diastole, continuously irritating the primary inflammatory area of the parietal endocardium, finally resulted in the formation of the pockets. The question arises whether the pockets were formed during the process of organization of the circumscribed parietal endocarditis, or whether they were formed secondarily after the scar formation had been completed. It is conceivable that as soon as the insufficiency of the valve was established, and as soon as irregularities were formed along the course of the regurgitating blood and pressure, the irregularities provided a foothold for the regurgitating blood which, with oft repeated insults to these areas, finally led to the formation of pockets. These irregularities might be either an organizing exudate or circumscribed endocardial thickenings, *i. e.*,

end stages of a parietal endocarditis. In many sections of the cusps of the pockets, blood vessels were seen extending from the endocardium through the bases of the pockets into the cusps. In addition, the cusps also showed inflammatory cells and blood pigment. These findings speak more for the fact that the formation of the pockets occurred during the period of organization. If the pockets had been formed secondarily upon an endocardial scar after the inflammation had subsided, we would rather expect to find in the cusps of the pockets hyalinized connective tissue without blood vessels or remnants of inflammatory exudate, mere evidence of mechanical irritation. I believe, therefore, that the regurgitating blood and pressure in aortic insufficiency produced the pocket formation in the area which was the seat of a parietal endocarditis undergoing organization. The finding of remnants of muscle fibers, with marked atrophy but without signs of inflammation in some portions, showed that the atrophy was more likely the result of a continuous mechanical pressure which brought about the formation of pockets, than evidence of past inflammations extending into the myocardium.

Endocardial Pockets in Hearts Showing Syphilitic Involvement of the Aortic Valve

Both of our cases showed pockets which were open in the direction of the aorta. But in addition, each case showed one pocket which was open in the direction of the apex of the heart. The sections of the pockets open toward the aorta showed an abundance of connective tissue with hardly any nuclear elements, no blood vessels, but many elastic fibers. A primary inflammation and secondary organization therefore can be ruled out. The only explanation I can offer for the formation of these pockets lies in the aortic regurgitation. The degree of insufficiency of the aortic valve in both cases was very marked. It must be assumed that the regurgitating blood and pressure acting as a chronic irritant primarily produced circumscribed fibrosed areas of the endocardium. As soon as irregularities of the endocardium were formed, the continuous regurgitation with formation of eddies in these regions finally resulted in the formation of pockets.

The pockets which were open toward the apex were found below the left aortic cusp very close to white, fibrosed areas in both cases.

This location of such pockets was commonly encountered by most of the investigators. Histologically none of the pockets showed remnants of an acute inflammatory exudate. In both instances they revealed only connective tissue which was poor in nuclei. Ribbert believes that the thickened endocardium in such areas was due to an extension of the syphilitic process from the valves to the endocardium. But these areas showed histologically nothing characteristic of syphilis. Libman,[17] in discussing Cohn's paper, does not believe in the syphilitic origin of endocardial pockets. Krasso believes that primary endocardial thickening, similar to that preceding the formation of pockets open toward the aorta, is due to the continuous pressure of the regurgitating blood in aortic insufficiency. He states that in the case of a relative stenosis of the conus arteriosus sinister, such thickened areas were secondarily transformed into pockets by the force of the systolic pressure. The location of such pockets just below the aortic valve, however, makes it seem unlikely that they were primarily the result of mechanical pressure of the regurgitating blood. Furthermore, there are cases reported, without evidence of insufficiency of the aortic valves, showing such pockets. Case 5 of this series, similarly did not disclose an aortic regurgitation.

The following table is offered to show the reported cases in which mention was found of pockets open toward the aorta. The table gives the name of the author, the diagnosis of valvular disease, the number of the particular case, and the number of pockets.

TABLE I

Case Reported with Pockets open Toward the Aorta

Author	Main diagnosis	Case No.	Number of systolic pockets
Wilke	Stenosis of the aortic orifice due to a papillary tumor of the aortic valve.	3	Several
Wilke	Recurrent endocarditis of the aortic valve. Stenosis of the aortic orifice and insufficiency (?) of the aortic valve.	4	Several
Kaewel	Syphilitic involvement of the aortic valve. Insufficiency of the aortic valve.	6	2
Kaewel	Syphilitic involvement of the aortic valve. Insufficiency of the aortic valve.	7	1
Kaewel	Syphilitic involvement of the aortic valve. Insufficiency of the aortic valve.	8	2
Kaewel	Acute, superimposed on chronic endocarditis of the mitral and aortic valves.	21	3
Böger	Healed thrombo-endocarditis, ulcerosa lenta. Stenosis of the aortic orifice and insufficiency of the aortic valve.	7	2
Böger	Healed rheumatic endocarditis of mitral valve.	12	1
Krasso (first paper)	Recurrent malignant endocarditis. Insufficiency of the aortic valve and moderate stenosis of the aortic orifice.	1	1
Krasso (second paper)	Healed endocarditis of the aortic and mitral valves. Insufficiency of the aortic valve and stenosis of aortic orifice.	1	1
Krasso (second paper)	Recurrent malignant endocarditis. Insufficiency of aortic valve and stenosis of aortic orifice.	2	1
Krasso (second paper)	Recurrent verrucous endocarditis. Insufficiency of aortic valve and stenosis of aortic orifice.	3	1
Krasso (second paper)	Syphilitic involvement of the aortic valve. Insufficiency of the aortic valve.	5	2
Krasso (second paper)	Healed endocarditis of aortic valve. Insufficiency of aortic valve.	6	1
Krasso (second paper)	Healed endocarditis of aortic valve. Syphilitic involvement of the aortic valve. Insufficiency of aortic valve.	8	Several

Seven cases out of the fifteen shown in the table were the seat of an unquestionable stenosis of the aortic orifice. Five cases showed a syphilitic involvement of the aortic valve; but the hearts of these cases were the seat of a marked hypertrophy and dilatation leading, as Krasso specifically pointed out, to a relative stenosis of the aortic conus. In his case (No. 6) showing an insufficiency of the aortic valve without organic stenosis of the orifice, Krasso emphasized the presence of a relative stenosis of the aortic conus. In Kaewel's case (No. 21) showing an acute, superimposed on chronic endocarditis of the aortic and mitral valves, the diagnosis of stenosis of the aortic orifice was not mentioned, but the hypertrophy and dilatation of the heart was emphasized. Böger's case (No. 12) showed a healed rheumatic mitral endocarditis. But neither the size nor the weight of the heart was given, so that nothing can be said about a possible relative stenosis of the aortic conus. Both of our cases of syphilitic involvement of the aortic valve, in which pockets open toward the apex were found, were the seat of a relative stenosis of the aortic conus brought about by the marked hypertrophy and dilatation of the heart. It is possible that the friction of the systolic blood stream, and pressure, is sufficient to produce a mechanical irritation of an area situated in the region of the stenosed conus. At the same time, the continuous impulse of the systolic blood stream, and pressure, might result in the formation of pockets. Krasso calls pockets which are open toward the aorta diastolic pockets, and those open toward the apex, systolic pockets. It seems that this nomenclature is justifiable and should be adopted.

Endocardial Pockets in Rheumatic Endocarditis

Case 5, which showed a healed rheumatic endocarditis resulting in a stenosis of the aortic orifice, presented only one systolic pocket just below the aortic valve area. However, the surrounding endocardium was diffusely thickened. The sections of both, the cusp of the pocket and the surrounding endocardium, revealed no indications of organization or remnants of an inflammatory exudate. The sections showed only fibrous tissue with a few nuclear elements. The aortic leaflet of the mitral valve showed no changes. This pocket, similar to the systolic pockets of the last two cases, was apparently the result of the continuous irritation and friction of the systolic

blood stream and pressure upon the area below the stenosed aortic orifice, producing, first, simple thickenings of the endocardium with secondary formation of pockets. The pathogenesis of the systolic pockets of this case and of the last two cases is apparently identical.

Sections of the auricular pockets of the second case of this group showed the presence of inflammatory cells, lymphocytes, polymorphonuclear leukocytes, endothelial cells and a new formation of connective tissue. The surrounding portions of the myocardium showed similar inflammatory cells. It is evident that the primary changes were inflammatory in nature. As in the first group of cases, the impulse of the regurgitating blood directed upon an area of organizing parietal endocarditis of the left auricle, caused the formation of pockets after the insufficiency of the mitral valve was established.

This case is especially noteworthy because a search through the literature disclosed only one other case, described by Abbott,[18] which showed pockets in the left auricle. This author found a thick-walled endocardial pocket in the left auricle of a heart which was the seat of a large open foramen ovale and button-hole stenosis of the mitral orifice. The depths of this pocket lay in close contiguity to a muscular channel running from an accessory chamber in the right auricle. The auricular endocardium was greatly thickened. The histological details, however, are lacking and it is, therefore, difficult to decide whether this pocket was inflammatory in origin or evidence of another malformation of the heart.

The various pockets in our cases, therefore, seem of different origin. Some are primarily inflammatory in nature, results of organizing parietal endocarditis, while others seem to be the result of primary mechanical irritation. The formation of the pockets themselves, however, is in the final analysis, always caused by the force of either systolic or diastolic regurgitating blood columns and pressure.

In the discussion of the pathogenesis of diastolic endocardial pockets, the ultimate cause for their formation appears to be the regurgitation of blood and pressure. Wiggers [19] in his "Circulation in Health and Disease" states that in aortic insufficiency only a small volume of blood actually regurgitates, and that the essential dynamic disturbance is brought about not by the volume of blood which regurgitates, but by the regurgitation of pressure during diastole. However, more recently, Wiggers and Green [20] found that in

artificially produced aortic insufficiency, the total regurgitation under optimum conditions can equal 50 to 60 per cent of the normal tidal volume in the perfused heart. It seems unlikely that the regurgitating pressure in aortic insufficiency is able to act upon one circumscribed area of the endocardium and produce there, in time, diastolic pockets. It is more likely that the regurgitating pressure occurring without fluid movement would extend equally in the various directions. The only possible explanation of the pathogenesis of the formation of endocardial pockets lies in the assumption of a regurgitation of blood. The regurgitation of pressure might play an additional, but much less important rôle. Whether the actual friction of the regurgitating blood, or whether eddies formed by the regurgitating blood and directed upon a circumscribed portion of endocardium produce the chronic inflammation which is the basis for the formation of some of the pockets, cannot be decided.

All the sections revealed the presence of a great number of elastic lamellae. Some of the sections showed the direct continuation of the internal elastic lamellae of the endocardium into the pockets.

Wilke, as stated before, is of the opinion that the pockets are manifestations of functional adaptation. A similar view was more lately expressed by Borst. Functional adaptation, however, implies that the part involved adapts itself to new functional demands (Karsner [21]) and actually fulfills the demanded functions (Borst). The endocardial pockets, however, only resemble pockets of aortic valves. Even though they are brought about by the force of the blood stream and are often found to be multiple, yet they cannot have any marked function because they are small and hold only a very insignificant amount of blood. To fulfill a function, it would be necessary that the pockets be close enough together to allow their cusps to touch during diastole as aortic cusps do. If such pockets were found in an entire row just below the aortic valve, a teleologist might be justified in assuming them to be evidence of functional adaptation.

Summary and Conclusions

1. In two cases of subacute bacterial endocarditis of the aortic and mitral valves with insufficiency of the aortic valve, endocardial pockets with openings toward the aorta were found on the interventricular septum of the left ventricle. The initial lesion which

brought about the pocket formation was a circumscribed parietal endocarditis. The continuous regurgitation formed the pockets secondarily.

2. In one case of rheumatic endocarditis of the mitral valve with insufficiency of this valve, endocardial pockets were present in the left auricle. These pockets were open toward the mitral valve. They also were primarily inflammatory in origin and formed secondarily by the regurgitation after the insufficiency of the mitral valve had been established.

3. In two cases of syphilitic involvement of the aortic valve with insufficiency of this valve, endocardial pockets open toward the aorta were found. These pockets were caused primarily by the mechanical irritation of the regurgitating blood columns.

4. Two cases of syphilitic involvement of the aortic valve with insufficiency of this valve and marked stenosis of the conus arteriosus sinister, and one case of rheumatic endocarditis of the aortic valve with stenosis of its orifice, showed endocardial pockets on the interventricular surface of the left ventricle. These pockets were open toward the apex of the heart. They were brought about by the mechanical irritation of the systolic blood stream acting as a trauma upon the endocardium in the region of the stenosed portions.

5. Diastolic endocardial pockets are evidence in favor of the view of actual regurgitation of blood volume.

6. The nomenclature of "diastolic pockets" referring to those open toward the aorta and "systolic pockets" referring to those open toward the apex (Krasso) is justified.

7. Endocardial pockets cannot be regarded as manifestations of functional adaptation.

I am indebted to Prof. H. T. Karsner for his valuable suggestions.

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DESCRIPTION OF PLATES

PLATE 137

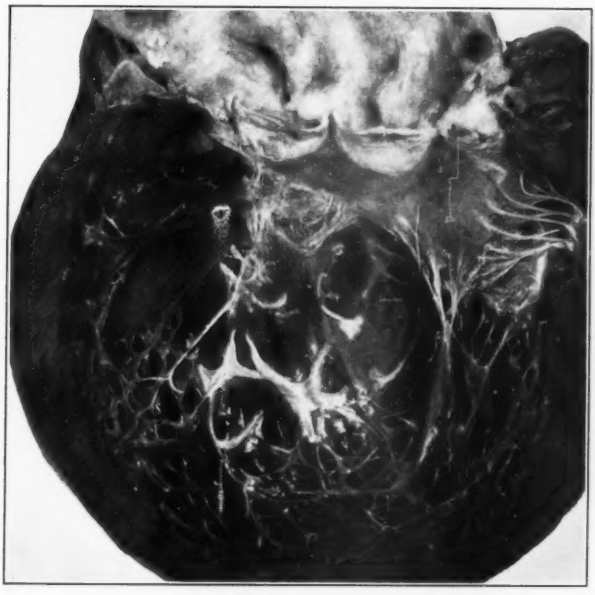
FIG. 1. Heart of Case 2. Diastolic pockets of the interventricular septum of the left ventricle.

FIG. 2. Heart of Case 3. Diastolic pockets on the interventricular septum and one systolic pocket below the left aortic cusp.

FIG. 3. Heart of Case 4. Diastolic pockets and one systolic pocket below the left aortic cusp. Note the marked dilatation of the heart.

1

2



3

PLATE 138

FIG. 4. Heart of Case 5. One systolic pocket.

FIG. 5. Heart of Case 6. Note two endocardial pockets on the left auricular endocardium.

FIG. 6. Cusp of pocket of Case 2. Note the inflammatory cells. Iron hematoxylin and eosin preparation. × 260.

FIG. 7. Section of base of pocket of Case 2. Note the inflammatory cells. Iron hematoxylin and eosin preparation. × 260.

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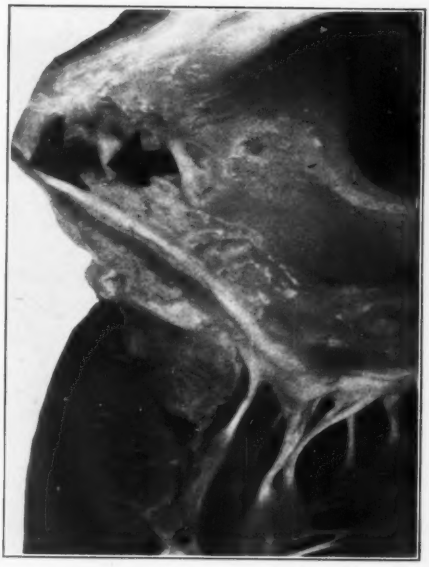

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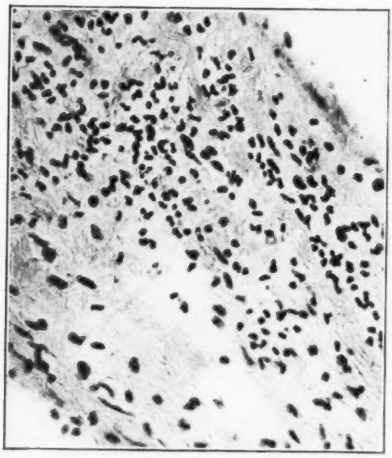

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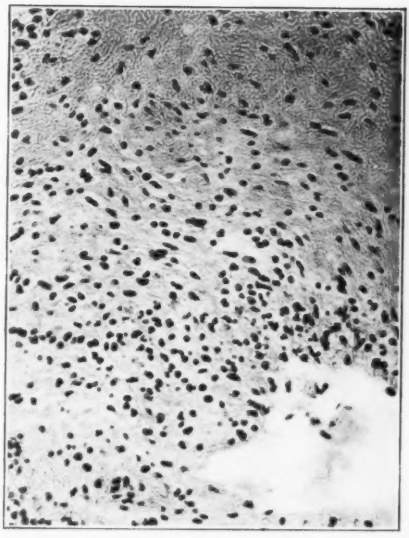


7

Saphir

Endocardial Pockets

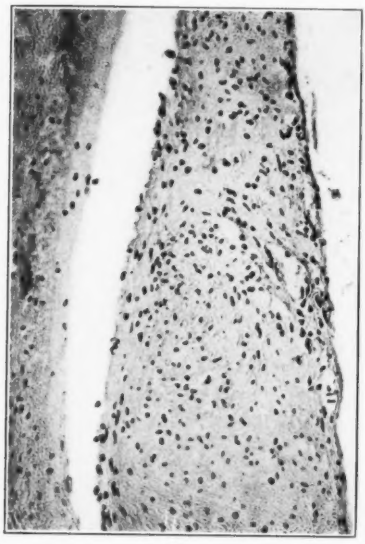
PLATE 139

FIG. 8. Cusp of pocket of Case 2. Note the newly formed blood vessels. Iron hematoxylin and eosin preparation. × 180.

FIG. 9. Fibrous band extending over one of the trabeculae carneae. Note the abundance of elastic lamellae. Orcein and iron hematoxylin preparation. × 80.

FIG. 10. Cusp of pocket and adjacent myocardium of Case 3. Note the spindle-shaped cells of the cusp and the atrophic muscle fibers. Inflammatory cells are not present. Iron hematoxylin and eosin preparation. × 180.

FIG. 11. Section of base of pocket of Case 6. Note the newly formed blood vessels. Iron hematoxylin and eosin preparation. × 180.



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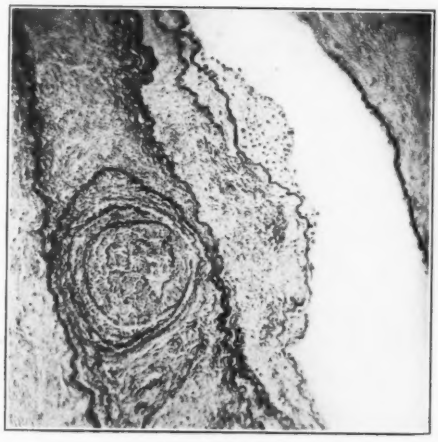

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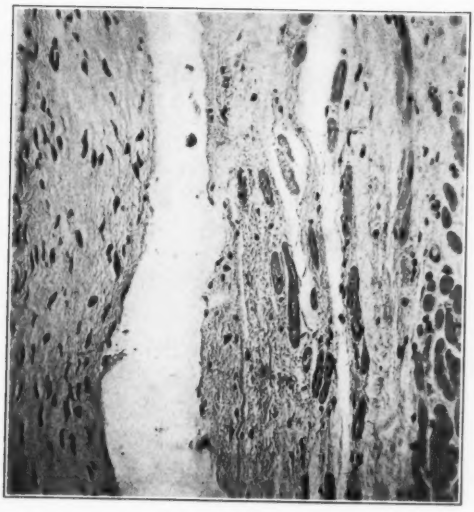

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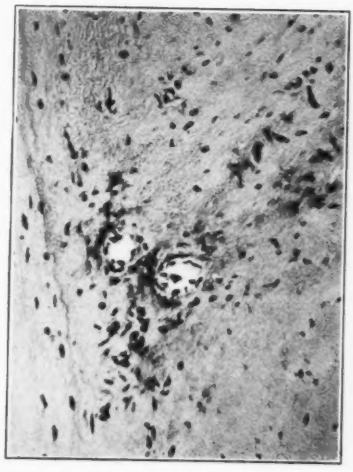

11

STUDIES IN TISSUE-IMMUNITY *

Cellular Reactions of the Skin of the Guinea Pig as Influenced by Local Active Immunization

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The relatively unsatisfactory results of many years emphasis upon humoral factors in the defense against infectious diseases have gradually led to a reëxamination of some of the underlying mechanisms involved, and as a result the cellular reactions of immunity are now receiving more attention. This is not surprizing in view of the many disappointments following attempts to secure preventive and curative serums, the transitory nature of passive immunity and the failure of protection at times in the presence of high concentrations of immune bodies in the serum. On the other hand, the permanency of active immunity, as after an attack of typhoid fever or small-pox, or from the administration of certain vaccines, offers possibilities of prevention far superior to those hitherto attained by the use of serums.

The emphasis upon cellular mechanisms of defense received renewed stimulus through the attention paid to the mesenchymal tissues by Aschoff [1] in the reticulo-endothelial system. The principal merit of Aschoff's work probably lies in its development of certain morphological aspects of defensive mechanisms; as a result, principles enunciated by Metschnikoff are now receiving more serious attention and are being revealed as fundamental in all considerations of the basic problems of immunology. Furthermore, accumulating evidence points increasingly to the close relationship between phagocytic cells, particularly macrophages, and antibody-formation, so that at present it is no radical concept that antibodies may be merely excess products resulting from the ingestion of antigenic substances by phagocytes. If this view is correct, the attempts to obtain serums or solutions of antibodies may be concerned mainly with by-products, the fundamental reaction actually occurring within the

* Received for publication July 21, 1930.

phagocytic cells. This does not imply that antibodies *per se* are unimportant; it does suggest the need of directing more attention to the cells themselves.

A further impetus to the enlargement of our concepts of tissue-immunity was furnished by Besredka [2] in his studies of local immunity. Although Besredka's interpretations of the mechanism of local tissue-immunity are not accepted by many, his ideas have stimulated much investigation and have aided in the clarification of a difficult problem of immunology. We also owe much of this clarification to the work of Gay and his associates,[3, 4, 5] who have greatly extended our knowledge of tissue-immunity and have done the most to correlate immune processes with histological changes in the tissues, thus furnishing a basis for the development of a field of histological immunology. In an extensive investigation of the problem of experimental streptococcic empyema, Gay and his collaborators have shown clearly that in this condition increased resistance of pleural cavities to streptococci is due primarily to an actual increase in numbers of tissue-macrophages in the wall of the thorax. Previous irritation of the pleural surfaces by the injection of such substances as gum arabic broth leads to the development or mobilization of large numbers of macrophages beneath the parietal pleura and these actively phagocytic cells ensure protection against large dosages when streptococci are later injected into the pleural cavity.

Opie [6, 7, 8, 9] has also made observations of the greatest importance in his studies of anaphylactic inflammation. He has demonstrated that when rabbits are injected intradermally with foreign proteins such as horse serum or crystalline egg albumin, much of the material is quickly demonstrable in the blood stream by precipitin tests, but "with repeated injection of the antigen the quantity of foreign protein that enters the blood stream diminishes and finally with advanced immunization none enters unless massive doses have been employed." Furthermore, in the immunized animal the foreign protein is fixed at the site of injection, a fact of significance because this is where the anaphylactic inflammation occurs. This inflammation is characterized by a rapid infiltration of polymorphonuclear leucocytes at the site of the inoculation, with edema and the deposition of fibrin. The small blood and lymph vessels are injured and thrombosis frequently occurs, leading, especially in the rabbit, to necrosis (Arthus phenomenon). Since a similar reaction occurs when antigen

and antibody are simultaneously injected into the tissues of a rabbit, but not when antigen and normal serum are so injected, and since the same effect is observed when antiserum is injected into the tissues of a sensitized animal, Opie concludes that "anaphylactic inflammation occurs because antigen and antibody have met in the tissues." If we assume that at least part of this union occurs within tissue macrophages, the intracellular reaction may conceivably either give no effect or may give anaphylactic inflammation, depending upon the amount of antigen entering sensitized macrophages in a short period of time, or the relative concentration of intracellular antibodies available for the reaction with the antigen. In either case a too violent reaction could be followed by a certain degree of damage to the surrounding tissues with injury to capillaries, thus increasing their permeability for the plasma and cellular elements so prominent in inflammation, anaphylactic or otherwise. It is a reasonable hypothesis that at least some of the cutaneous reactions which we regard as anaphylactic or allergic may be due to an altered reactivity of these phagocytic cells, both as to rate of phagocytosis and rate of intracellular digestion of antigenic substances, as determined by the number of phagocytes available, their state of reactivity and the amount of antigen utilized per unit of time. Where anaphylactic inflammation occurs, the extent of the cellular infiltration may depend upon the degree of the chemotactic stimulus; at times the very violence of the reaction may overshadow the beneficent action, giving the "two-edged sword" effect of dissemination of the antigen.

To what degree can we regard this reaction as a mechanism of defense? The answer will be determined by the behavior of properly sensitized tissues to the entrance of pathogenic microörganisms. Probably the essential difference between the effects of infectious agents and of inert antigens is in the ability of the former to multiply and to invade tissues. If both multiplication and invasion could be prevented to a large extent because of a local union of antigen and antibody through an agglomerating reaction, such as agglutination or precipitation of the microörganisms or their products, the tendency to dissemination of the germs would be greatly lessened. If, in addition, an increased number of macrophages, especially ones sensitized by previous experiences with the antigen, were present in the area of invasion, phagocytosis should be quantitatively increased. Finally, if the reaction to injury through this local meeting

of antigen and antibody were followed by a secondary infiltration of cells of inflammation into this area, the infectious agents should be even more effectively localized. This entire mechanism should then be considered as cellular and a type of tissue-immunity. Even in the presence of marked necrosis at the site of inoculation, if such a local reaction prevents the dissemination of the infectious antigen, the effect nevertheless is protective and an evidence of immunity. As Opie says, "the apparent susceptibility of the protected animal to local injury is a paradox explained by changes which serve to protect the organism as a whole." In other words, a scar from a furuncle is a small price to pay for the prevention of pyemia.

A phase of tissue-immunity which has received much attention within recent years is the problem of local immunity. In regard to this, we feel that too much emphasis has probably been placed on the localized nature of the immunity and on the rôle of antibodies in the phenomenon. Besredka defines local immunity as an "immunity without the obligatory participation of antibodies." Such a conception, if correct, would materially modify the usual conceptions as to the relative importance of cellular and humoral factors in immunity. Besredka's ideas are based, principally, on the evidence of an acquired immunity of local tissues, such as the skin and mucous membranes, in the absence of any significant degree of antibody-concentration in the blood serum. This, however, does not necessarily exclude the possibility of antibodies being within the cells or around them in the area of localized tissue-immunity; indeed, there is evidence that local concentration of antibodies may play a considerable part in the local tissue-immunity. Gay's definition of local immunity as an immunity "due to a locally superior mechanism for the disposal of a particular microörganism," seems to be much broader and more in conformity with the facts. This mechanism may or may not be associated with the action of antibodies, but until we know what function antibodies have within phagocytic cells and how quickly they may appear there, we cannot arbitrarily exclude them from participation in immune processes merely because they may seem to be of little significance as shown by the usual serological tests, especially in view of the increasing evidence that the usual site of antibody formation is in the individual cells of the mesenchymal tissues.

The demonstration of a localized type of immunity does not neces-

sarily imply the absence of protection elsewhere; resistance locally as well as generally is relative and a matter of degree. Nevertheless, by proper dosage, and by localized immunization, several investigators have shown that certain tissues may acquire an enhanced ability to resist invasion by microörganisms as compared with other tissues of the same individual. In such conditions of local immunity more evidence is needed as to the factors concerned, whether cellular, humoral, or both. Morphological evidence, particularly, is desirable, either of local dissolution or agglomeration of bacteria injected, or of an increased number of phagocytic cells in the area, or of increased metabolic activity of such cells. Curiously enough, although Besredka speaks of certain "receptive cells" which are dominant in localized areas of immunity, it seems that he has made little study of them from a histological viewpoint; nor have most of the other workers in this field given much consideration to this point.

Apparently the only histopathological study of the cellular reactions of the skin of the guinea pig to staphylococcus infection according to the methods of Besredka, is that of Freedlander and Toomey.[10] These workers made a detailed examination of the inflammatory response in the subcutaneous tissues of normal guinea pigs, and of ones previously treated with broth compresses and staphylococcus filtrates prepared according to the methods of Besredka. A definite localized protection was observed following the application of broth compresses, which protection persisted longer than twenty-four hours and less than seven days. The protection seemed to be non-specific and was correlated with histological changes in the subcutis where there was a significant increase in the numbers of clasmatocytes, fibrocytes and lymphoid cells following the application of sterile broth compresses for forty-eight hours. The inflammatory response to the subcutaneous injections of staphylococcus cultures in such animals was characterized by an infiltration of cells of inflammation in the subcutis, much more marked in degree than in normal animals similarly infected. Also, although polymorphonuclear leucocytes were the predominant cells in each case, they tended to degenerate in the control animals, whereas they retained their normal appearance in the broth-protected ones. In addition, in the latter animals there was an increased infiltration of small mononuclear cells and a greater prominence of clasmatocytes, with fibroblasts tending to organize the process at an early

stage. The authors concluded that "the clasmatocytes in large numbers diminish the virulence of the bacterial attack and also, by ingesting the polymorphonuclear leucocytes which contain staphylococci, they prevent a recurrence of bacterial activity."

An important study of the inflammatory reactions of the subcutaneous tissues of normal and immunized mice, using streptococci and pneumococci, was also made by Tsuda [11] in Lubarsch's laboratory. In normal animals this response varied with the degree of virulence of the microörganisms injected, but with weakly virulent germs the organisms remained at the site of inoculation, were quickly surrounded and phagocytosed by leucocytes and then encapsulated by connective tissue cells. There was thus no dissemination of the microörganisms through the adjacent tissues. With highly virulent microörganisms, however, there was an early injury to the tissues at the site of inoculation with very little phagocytosis of the germs, and with a consequent rapid dissemination of the latter throughout the surrounding tissues. In immunized animals both virulent and avirulent bacteria were quickly injured, as shown by evidences of degenerative changes such as swollen and poorly stained forms, inequality of size, etc. There was also a marked tendency to agglutination followed by active phagocytosis by the leucocytes and macrophages. This observation of agglutination *in vivo*, while not emphasized by Tsuda, is obviously of great significance. Tsuda states that "if the immunity is strong enough, the injected cocci show at the site of injection agglutination phenomena in the form of floccular clumps and aggregations of microörganisms."

More recently Imschenetzky [12] has shown that the application to rabbits of dressings saturated with isotonic salt solution or with staphylococcus antivirus solutions leads to a distinct inflammation in the subcutaneous tissues, with hyperemia, edema and an increased prominence of histiocytes and increased numbers of infiltrated leucocytes. Similar dressings saturated with a 1 per cent solution of trypan blue in salt solution led to the appearance of granules of dye in the histiocytes, but only after forty-eight-hour application of the dressings, and then only when there were evidences of slight injuries to the epidermis which had increased its permeability.

Experimental Procedures

Our studies have been concerned with the cellular reactions of defense in the skin of normal guinea pigs and of others previously immunized by the intracutaneous injection of a staphylococcus vaccine. More than 100 different animals have been observed during the course of the investigation. A strain of staphylococcus aureus freshly isolated from a furuncle was used. For immunization, a twenty-four-hour growth on agar slants was suspended in 1 cc. of sterile 0.9 per cent salt solution and heated at 60° C for one hour. Two-tenths of a cubic centimeter of this vaccine was injected intradermally at daily intervals for ten days into the anterior abdominal wall of guinea pigs weighing from 200 to 300 grams, thus infiltrating an area of skin of approximately four square centimeters. The animals were then allowed to rest for twenty-five days in order to permit the skin to return to approximately normal conditions in so far as external appearances were concerned. Then these animals, and normal ones of the same size, were injected intradermally with 0.2 cc. of a living virulent culture of the organism, the growth from one agar slant again having been suspended in 1 cc. of sterile salt solution.

At intervals the guinea pigs were anesthetized and the areas of inflammation were excised after attaching the peritoneal surface to a cork frame by means of bamboo pegs. The tissues, usually averaging from one to one and a half centimeters in width, were immediately fixed in formol-Zenker fluid and subsequently embedded in celloidin and sectioned at 10 microns. The sections were stained routinely with Maximow's hematoxylin-eosin-azur II as well as with special stains such as Mallory's connective tissue stain, Goldmann's carmine stain and Gram's stain.

Effects of the Intradermal Injection of a Staphylococcus Vaccine upon the Skin

Sections of skin taken twenty-five days subsequent to the ten intradermal inoculations of killed staphylococcus vaccine show the principal effect in the subreticular zone of the subcutis. Here there is an extremely marked increase in macrophages. In the looser areas many sizes and types of non-granular cells may be seen, varying from typical lymphocytes and monocytoid forms to typical macrophages.

In the denser areas the latter are elongated and compressed and at times resemble fibroblasts. Mallory's connective tissue stain, however, shows but slight increase in the amount of collagen, although there is a definite increase of collagen in the densest areas of macrophages. Mitotic figures are not seen in the regions of thickening, and the multiplicity of mononuclear types, from lymphocytes to typical macrophages, suggests that, as maintained by Maximow, the latter may be differentiated forms of cells of hematogenous origin, particularly lymphocytes or monocytes (see Fig. 1).

The Behavior of India Ink Injected into Normal Skin and into Skin Previously Immunized against Staphylococci

The intradermal injection of 0.2 cc. of a 4 per cent suspension of India ink in sterile isotonic salt solution into a normal guinea pig was followed by a diffuse dispersion of the particulate material through the subcutis. Sections showed much of the ink caught along the collagenic fibrils, although some of it was engulfed by tissue macrophages. A moderate infiltration of polymorphonuclear leucocytes occurred at the end of twenty-four hours, but these did not engulf the particles of ink to any extent. When the same quantity of ink was injected into an area of skin of a guinea pig which had previously been given ten intracutaneous injections of plain peptone broth, the effects were not noticeably different from those observed in the normal animal. When a similar quantity was injected, however, into the skin of a guinea pig which had previously been immunized by ten intradermal injections of the killed staphylococcus vaccine, there was a distinct tendency for the ink to remain localized near the site of inoculation rather than to be dispersed through the subcutis. Fig. 9 shows the distribution of the ink in the skins of the three animals and Fig. 2 illustrates the mode of disposal of the ink particles by the macrophages of the subcutis. It is interesting that monocytoid cells, lymphocytes and polymorphonuclear leucocytes show little tendency to engulf the particles of ink. These observations suggest that merely the presence of increased numbers of macrophages increases quantitatively the engulfment of particulate material and thus effectively aids the localization of such material after its inoculation. It is also possible that local hindrances to lymph flow may further prevent, to some extent, the dissemination of the particulate material.

General Results

The inflammatory response to the living staphylococci was markedly different in the skins of the two groups of animals. In the normal guinea pigs the intradermal inoculation led to a serosanguineous inflammation which spread as a diffuse cellulitis through the subcutaneous tissues and frequently led to the death of the animal in from eighteen to twenty-four hours. In the previously immunized ones, however, the intradermal injection was followed by a localized small area of suppuration which tended to ulcerate and heal with no serious consequences to the host. It is evident from these differences in the reactivity that the intradermal injections of the killed culture of staphylococcus led to an increased resistance of the skin to the later injection of the living virulent organisms. Examination of the tissues confirmed the above observations and in addition suggested an explanation for the increased resistance of the skin of the immunized animals.

Effects of Intradermal Injections with Living Staphylococcus Aureus Suspension upon Normal Guinea Pigs

The inflammatory response is well developed within six hours, as shown in Fig. 5*a*. The principal finding in the skin of the normal animal at this stage is edema of the subcutis with separation of the collagenic fibrils and a beginning infiltration of cells of inflammation. Polymorphonuclear leucocytes comprise the vast majority of the incoming cells and these are actively phagocytosing the staphylococci as shown in Fig. 3. In spite of this fact, however, the staphylococci are diffusely spread along the subcuticular tissue in the form of a developing cellulitis. There is no evidence of any localizing tendency of the microörganisms, as shown in Fig. 10, nor are there evidences of injury to the bacteria, such as numerous swollen or distorted forms or frequent Gram-negative cocci. The infection is predominantly dispersive and generalizing. In the later stages, twelve, eighteen, and twenty-two hours, the picture is similar except for the greater infiltration of cells of inflammation, principally polymorphonuclear leucocytes (Figs. 6*a*, 7*a* and 8*a*). In spite of the activity of the microphages in ingesting many staphylococci, the infection progresses; in other words, the natural resistance is inadequate as a defensive mechanism.

Reactions in Skin Previously Immunized by Intradermal Injections of a Killed Staphylococcus Vaccine

The inflammatory response to the injection of 0.2 cc. of the same suspension of living staphylococci into the skins previously immunized is markedly different. As may be seen in Figs. 5*b*, 6*b*, 7*b* and 8*b* the principal difference is one of degree. At the six-hour stage there is an enormous infiltration of cells of inflammation in the subcutis of the immunized skin, much more abundant than in the normal animal at this stage. Furthermore, there is a qualitative difference in that there are many more lymphocytes and monocytoid cells present. These cells tend to become massed around a region of marked bacterial concentration and here there are many evidences of necrosis of the cells of inflammation, with accompanying hemorrhage and even thrombosis of the capillaries. Outside this area there are very few staphylococci to be seen; the infection is definitely localized and non-dispersive. A point of interest and probably of great importance is that in the area where the staphylococci are massed they are not present as individual organisms, but occur extracellularly in clumps and clusters, large and small, even in the six-hour stage. The appearance is that of agglutination *in vivo* (Figs. 11 and 12). It is around these clumps of staphylococci that the infiltration of leucocytes is densest, with the microphages nearest to the bacteria containing large masses of microörganisms. Here also the macrophages contain countless numbers of cocci, as shown in Fig. 4. It is obvious that the infection is localized to the immediate vicinity of the site of inoculation and that the acquired resistance is adequate as a defensive mechanism.

Discussion

It is an interesting fact that Metschnikoff showed in 1884 [13] that the principal difference in the reaction to the subcutaneous injection of virulent anthrax bacilli in normal rabbits, and in others previously immunized, lay in the greater degree of phagocytosis in the latter. He noted that within a few hours after the injection of the organisms into the normal animal there was an exudation rich in fluid and poor in leucocytes, in spite of the fact that the blood vessels in the vicinity were distended with blood and therefore could not be considered as unable to bring the leucocytes to the infected area. In the

vaccinated rabbits, however, there was an exudate rich in leucocytes at the site of inoculation and these were actively phagocytosing the bacilli. Metschnikoff concluded that the essential difference depended upon the sensitiveness of the leucocytes which exhibited a negative chemotaxis in the normal rabbit, but a marked positive chemotaxis in the immunized ones.

The effects of the entrance of pathogenic bacteria into the skin will obviously depend upon their ability to gain a foothold, multiply and disseminate throughout the body. There is no doubt that microorganisms vary in their ability to adapt themselves in the animal's body, this probably being a property inherent in the microörganisms themselves. The growth energy of certain highly virulent strains may possibly be so pronounced at times that dissemination occurs before the body cells or fluids can mobilize to hinder this dissemination.

Under more usual conditions the ability to adapt, multiply and disseminate is prevented by the defensive forces of the body. These may be both cellular and humoral. The early mobilization of phagocytic cells at the site of bacterial infection may serve to restrain the rapid increase in numbers of bacteria and in most cases the rate of engulfment by the macrophages may exceed the rate of multiplication of the bacteria. This, plus probable mechanical hindrances from the accumulation of fibrin and cells around the region of infection, will effectually localize the latter. In any event, the infection is obviously localized and the dissemination of the microörganisms is prevented.

In this localization of the bacteria at the site of inoculation the exact mechanism is still somewhat obscure. Are the organisms localized because of the intense infiltration of cells and fluids which mechanically hinder the further spread of the bacteria in the sense of the allergic inflammation of tuberculosis as conceived by Krause,[14] or are the organisms first localized by a mechanism of immunity and secondarily encapsulated because of the infiltration of cells of inflammation? The work of Opie would suggest the latter explanation as better fitting the facts and our results indicate the same probability. For example, the demonstration in the immune guinea pig within six hours after the injection of distinct extracellular clumping of the staphylococci with an accompanying failure of dissemination of the microörganisms strongly suggests a primary localiza-

tion of the bacteria through their reaction with antibody. Furthermore, no such tendency at any stage was noticed in the normal animals so that it does not seem probable that the bacterial masses are colonies growing in the tissues. It is of course possible that the clumping of the bacteria in the immune animal may be due to mechanical interferences with lymph flow which, with the dense layer of macrophages surrounding them, may keep the staphylococci localized. This conception seems less probable, however, when one sees how easily the polymorphonuclear leucocytes infiltrate the area and surround the masses of bacteria. We suggest, rather, that there is an actual antigen-antibody reaction in the tissues; as a result of this reaction chemotactic substances are formed which quickly lead to a pronounced infiltration of cells of inflammation, which are both quantitatively and qualitatively different from those responding in the normal animal (anaphylactic inflammation). Added to this, also, is the evidence that phagocytosis by the histiocytes is more abundant quantitatively than in the normal animals, in addition to the greatly increased number of histiocytes available in the former. The experiments with India ink, described above, strongly suggest hat an increase in histiocytes alone tends to localize particulate materials, but whether this is the result of increased phagocytosis or of a mechanical barrier remains uncertain. In active infection, however, it is probably the summation of all of these forces, specific as well as non-specific, that ensures an effective resistance against extension of the infection.

Additional support to the conception of the specific reaction is furnished by the experiments of Mudd, Lucké, McCutcheon and Strumia,[15, 16] which show the correlation between agglutination, cohesiveness, opsonization and phagocytosis of bacteria. If such correlations also obtain within the body, the evidences of agglutination and phagocytosis in our experiments may furnish a further clue to the function of immune bodies. Agglutination of the staphylococci may have furthered the tendency to their localization near the site of inoculation; their coalescence into small masses may also have increased the defensive efficiency of the phagocytes, both leucocytes and macrophages, since more microörganisms per phagocyte may be ingested following chance contacts than if the organisms were single and dispersed. Furthermore, if the agglutinating tendency and increased cohesiveness have an opsonizing effect, phagocytosis

will be further increased. In this connection it is interesting to recall the earlier conceptions of Bull [17] in his statement that "the degree of agglutination and opsonization of bacteria within the animal body is inversely parallel to the infectiousness of the bacteria for the host."

The relative importance of non-specific and specific agencies in localized tissue-immunity is difficult to evalute. Certainly non-specific factors may be sufficient to protect against many multiples of the lethal dose of an infectious agent, as was well shown in the experiments of Gay and his collaborators in the study of streptococcic empyema. Rivers and Tillett,[18] and Mallory and Marble,[19] found that the injection of plain meat infusion broth protected the skin of rabbits against later injections of streptococcus and staphylococcus. Miller [20] also demonstrated a definitely increased resistance of the skin of guinea pigs to staphylococcus, and of rabbits to streptococcus infections following previous treatments with dressings saturated with bouillon and peptone water. In all of these experiments, however, the results do not prove that such non-specifically increased resistance would have been adequate for larger infective doses of the microörganisms used, or that even better protection might not have been secured by the aid of specific modes of treatment.

The mechanism of the non-specifically increased resistance may be explained in at least two ways: first, in a local infiltration of leucocytes and bactericidal substances which may more effectively dispose of the infecting organisms later injected, or second, in a stimulation of the local fixed-tissue cells to increased functional activity. Evidence for the second possibility is suggested by Katsunuma and Sumi [21] in their observation that when rabbits were injected subcutaneously with a suspension of staphylococci on one side, and with a similar quantity of salt solution on the other side, followed a few hours later by an injection of an emulsion of staphylococci into both sides, the exudates collected showed much more active phagocytosis on the side previously injected with the staphylococci.

The evidence is quite convincing, however, that non-specific factors are not exclusively responsible for the increased resistance in localized tissue-immunity. For example, Gay and his associates found in testing the protection of the pleural cavity to streptococcus infection that "the degree of protection acquired by the repeated administration of living streptococci subsequent to broth or aleu-

ronat preparation of the cavity is markedly increased over the protection obtained by a single injection of the broth or aleuronat." More recently Clark [22] has shown the importance of specific mechanisms in experiments concerning tissue-immunity to pneumococcus infections of the pleural cavity of rabbits. With this organism no protection was obtained following the injection into pleural cavities of substances which had previously been shown to protect completely against infection with the streptococcus through the mobilization of macrophages. When the pneumococci, however, were treated with immune serum before being injected into the prepared pleural cavity, there was marked protection in cavities containing an exudate rich in mononuclear cells and with a pleural wall fortified by increased numbers of macrophages. These experiments clearly show the significance of specificity; the opsonization of the organisms is thus an important feature which favors increased phagocytosis by the macrophages.

The specific factor in our experiments seems to be mainly an agglomerating force which we believe is true agglutination *in vivo*. Certainly the microörganisms occur in clumps in the immune skins and are not thus seen in the normal skins. It is possible that the increased number of macrophages present in the immunized skins interferes with lymph flow in a mechanical fashion and thus encourages approximation of groups of cocci; experiments now in progress may throw further light on this phase of the problem. If we assume that the agglomeration of the staphylococci is specific agglutination *in vivo*, we have concrete evidence of a fundamental immunological rôle of such antibodies in aiding the local fixation of antigen in tissues. Opie's experiments clearly prove this for precipitins and he has shown that such precipitates are strongly chemotactic for polymorphonuclear leucocytes which probably destroy the injected antigen by intracellular digestion. We believe that this concept is of the utmost importance when applied to the fate of living antigen introduced into the tissues. In our experiments the facts are clear that the staphylococci disseminate diffusely throughout the subcutaneous tissues of the normal animals with no tendency to agglomerate or agglutinate. On the other hand, in the immune animals the tendency to agglomerate is noticed as early as six hours after injection of the microörganisms. Coincidently, there is a localization of the staphylococci near to the site of

inoculation, with a pronounced infiltration of cells of inflammation, both granulocytes and agranulocytes, around the bacteria. Local injury to this area occurs, but the animal itself is protected. In the words of Opie "vital organs are protected at the expense of local injury."

Summary and Conclusions

This paper describes histopathological studies of the skin and subcutaneous tissues of the abdominal wall of normal guinea pigs and of ones previously immunized by intracutaneous injections of a staphylococcus vaccine, all infected by the intracutaneous injection of a live virulent culture of staphylococcus aureus. The inflammatory responses were markedly different in the two groups. In the normal animals the inflammation was characterized mainly by an infiltration of polymorphonuclear leucocytes which actively phagocytosed the microörganisms. In spite of this the staphylococci showed no tendency to localize, but disseminated throughout the subcutaneous tissues in the form of a cellulitis.

In the previously immunized animals, however, the staphylococci tended to remain localized near the site of inoculation where they were seen agglomerated in bacterial masses of various sizes, presenting the picture of a genuine agglutination *in vivo*. Coincidently the infiltration of cells of inflammation led to further localization of the microörganisms so that only a localized area of necrosis resulted.

The previous immunization by intracutaneous injections of the staphylococcus vaccine was followed by a marked thickening of the subreticular layer of the subcutis, due mainly to increased numbers of tissue macrophages having been either activated or produced. Evidence is presented that many of these are derived from agranulocytes of the blood. These macrophages were actively phagocytic for the live staphylococci and furnished an effective barrier against extension of the infection.

The immunity secured by the above procedures is predominantly cellular in type with the tissue-macrophages playing the dominant part, due to increased numbers and also probably to increased metabolic activity. In addition, localization of the microörganisms by the action of agglutinating or opsonizing antibodies is suggested as of primary importance in preventing the dissemination of the infectious agent. The combination of humoral and cellular mechanisms ensures an adequate resistance against the bacterial invaders.

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DESCRIPTION OF PLATES

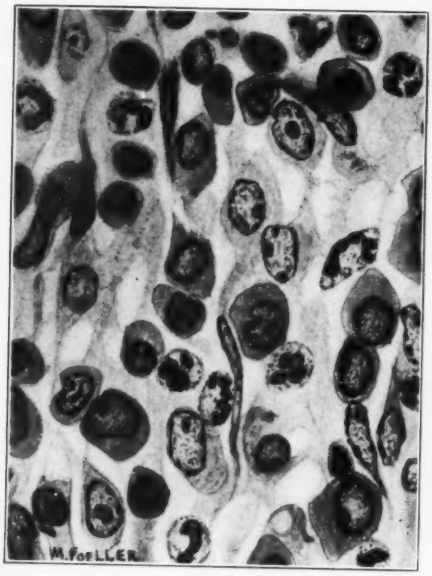
PLATE 140

FIG. 1. Drawing made at the level of the substage with the aid of the camera lucida, Leitz Oc. 4, Obj. 2 mm. apochromatic, showing the effects of intracutaneous injections of a staphylococcus vaccine upon the subcutis of a guinea pig. Note the types of agranulocytes, varying from typical lymphocytes and monocytoid forms to typical macrophages. The tissue was excised twenty-five days after the end of the period of immunization. Stained with hematoxylin-eosin-azur II.

FIG. 2. Oil immersion drawing made at the level of the substage with the aid of the camera lucida, Leitz Oc. 4, Obj. 2 mm. apochromatic, stained by Goldmann's carmine method. This illustrates the mode of disposal of particulate material by macrophages in the subcutis of the guinea pig after intracutaneous immunization by ten injections of staphylococcus vaccine. This animal was injected intradermally with 0.2 cc. of a 4 per cent suspension of India ink in 0.85 per cent sodium chloride solution, and the skin excised twenty-four hours later. Note the active ingestion of ink particles by the macrophages and the absence of such ingestion by the lymphocytes, polymorphonuclear leucocytes and monocytoid cells. Note also the adherence of small particles to fibrils of collagen.

FIG. 3. Oil immersion drawing, made at the level of the substage with the aid of the camera lucida, Leitz Oc. 4, Obj. 2 mm. apochromatic, stained with hematoxylin-eosin-azur II. This drawing is from the subcutis of a normal guinea pig six hours subsequent to the intracutaneous injection of 0.2 cc. of a living virulent suspension of staphylococcus aureus. Note the active ingestion of staphylococci by the polymorphonuclear leucocytes, which cells are almost the only ones responding at this stage of the inflammation. Note also the diffuse distribution of these cells and of the microörganisms.

FIG. 4. Oil immersion drawing, made at the level of the substage with the aid of the camera lucida, Leitz Oc. 4, Obj. 2 mm. apochromatic, from section stained with hematoxylin-eosin-azur II. From the subcutis of a guinea pig previously immunized intracutaneously by injections of staphylococcus vaccine, and infected intracutaneously with 0.2 cc. of a living virulent suspension of staphylococci. Tissue excised eighteen hours after the infection shows many macrophages actively phagocytosing staphylococci. The infection remained localized to the site of inoculation.



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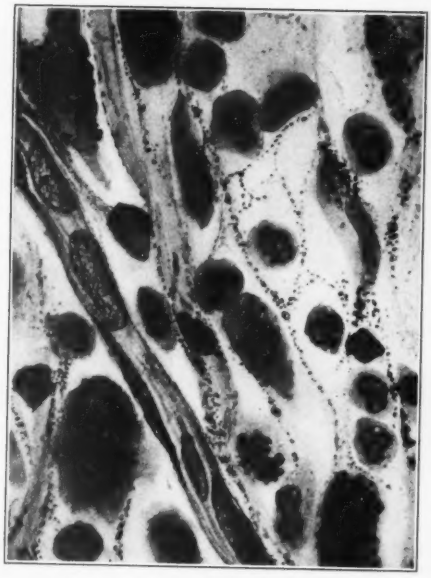

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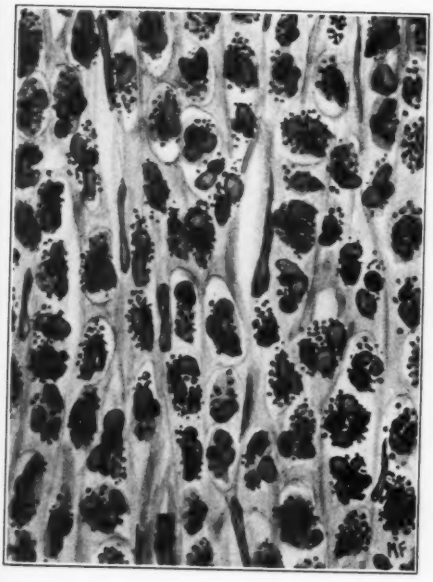

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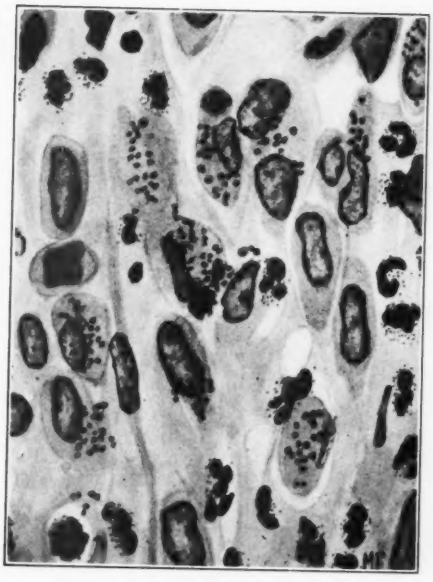

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PLATE 141

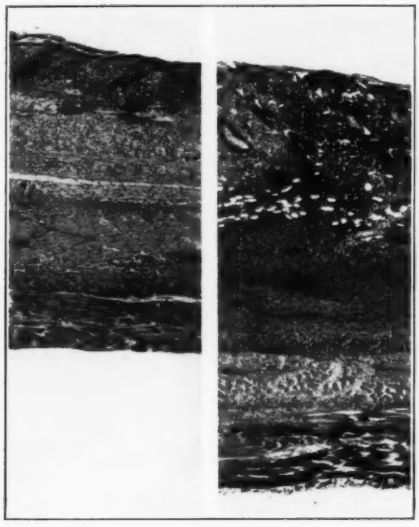
FIG. 5. Photomicrographs of sections through the skin and entire abdominal wall of (*a*) normal guinea pig and (*b*) intracutaneously immunized guinea pig. The tissues were excised six hours following the intracutaneous injection of 0.2 cc. of a living virulent suspension of staphylococci into each animal. In (*a*) there is slight edema of the subcutis with beginning infiltration of cells of inflammation. In (*b*) note the increased thickness of the subcutis and the more intense infiltration of cells of inflammation. × 25.

FIG. 6. Photomicrographs of sections through the skin and entire abdominal wall of two other guinea pigs treated as described in Fig. 5. The tissues were excised eleven to twelve hours following intracutaneous infection. × 25.

FIG. 7. Photomicrographs of sections through the skin and entire abdominal wall of two other guinea pigs treated as described in Fig. 5. The tissues were excised eighteen hours following intracutaneous infection. × 25.

FIG. 8. Photomicrographs of sections through the skin and entire abdominal wall of two other guinea pigs treated as described in Fig. 5. The tissues were excised twenty-two hours following intracutaneous infection. × 25.

Note in all instances the more intense inflammatory response in the subcutis of the immunized animals.



a b

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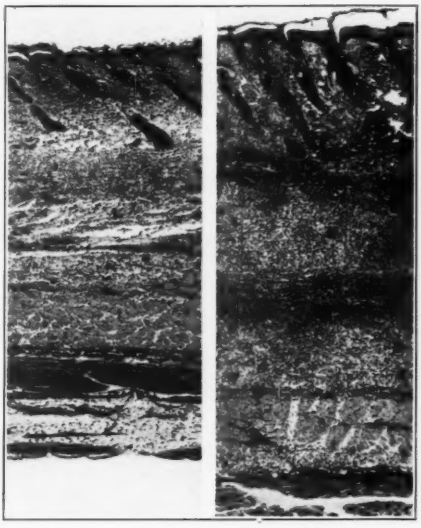

a b

6

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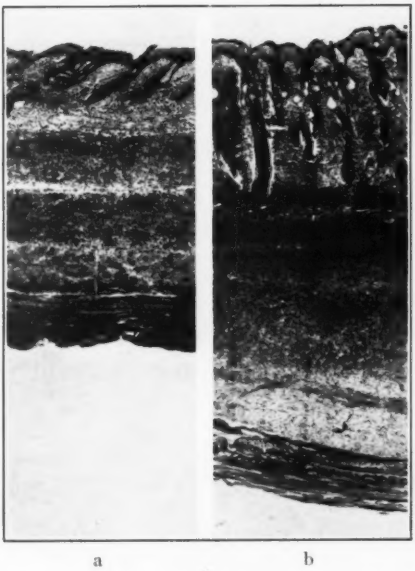
7



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8

Cannon and Pacheco Studies in Tissue-Immunity

PLATE 142

FIG. 9. Photograph of celloidin-embedded tissues of the entire thickness of the abdominal wall from three guinea pigs, each having been injected intracutaneously twenty-four hours before with 0.2 cc. of a 4 per cent suspension of India ink in 0.85 per cent sodium chloride solution. (*a*) Normal. (*b*) Previously given ten intracutaneous injections of sterile peptone broth. (*c*) Previously immunized by ten intracutaneous injections of the staphylococcus vaccine, the last injection given eleven days before. Note the dissemination of the suspension of ink along the subcutis in Nos. 1 and 2 and the tendency to localization of the ink near the site of inoculation in No. 3.

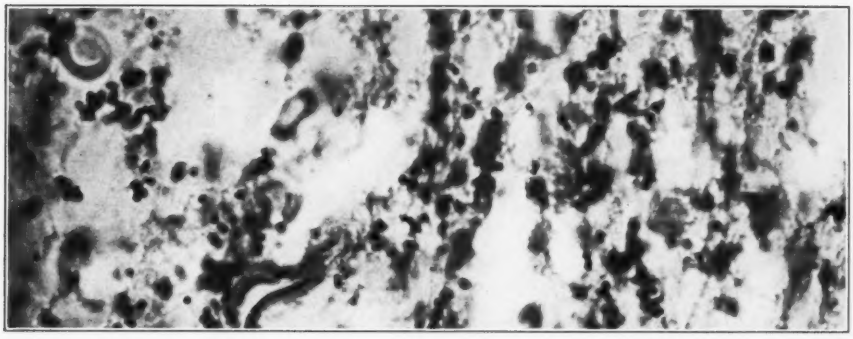
FIG. 10. Photomicrograph of the subcutis of a normal guinea pig six hours following the intracutaneous injection of 0.2 cc. of a living virulent suspension of staphylococcus aureus. Note the diffuse distribution of the microörganisms with the tendency to spread along collagenic fibrils and to occur singly or in small clusters. × 1400.

FIG. 11. Photomicrograph of the subcutis of a previously intracutaneously immunized guinea pig six hours following the intracutaneous injection of 0.2 cc. of the same suspension of staphylococci injected into the animal shown in Fig. 10. Note the greater tendency to concentration of the microorganisms, with the occurrence of the staphylococci extracellularly in coalescing clumps and clusters. The appearance is strongly suggestive of agglutination *in vivo*. × 1400.

FIG. 12. Photomicrograph of the subcutis of a guinea pig previously immunized intracutaneously, twenty-two hours following the intracutaneous injection of 0.2 cc. of a living virulent suspension of staphylococcus aureus. Note the large masses of extracellular staphylococci, in clusters large and small, suggesting agglutination *in vivo*. These masses were completely encircled by a dense accumulation of cells of inflammation, principally polymorphonuclear leucocytes and macrophages. There was no tendency for the infection to disseminate beyond this area of microörganisms. × 1400.

a b c

9



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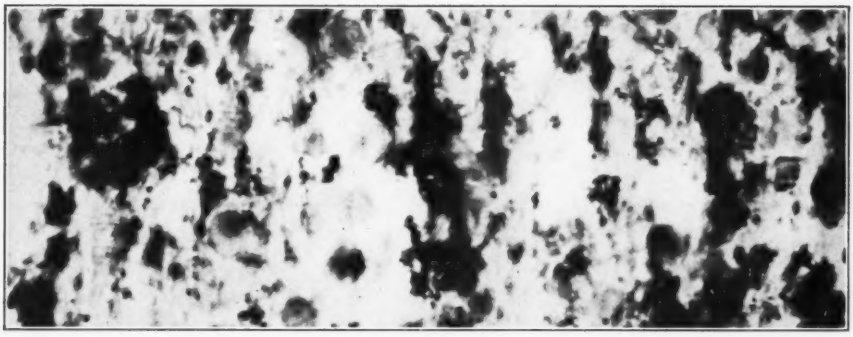

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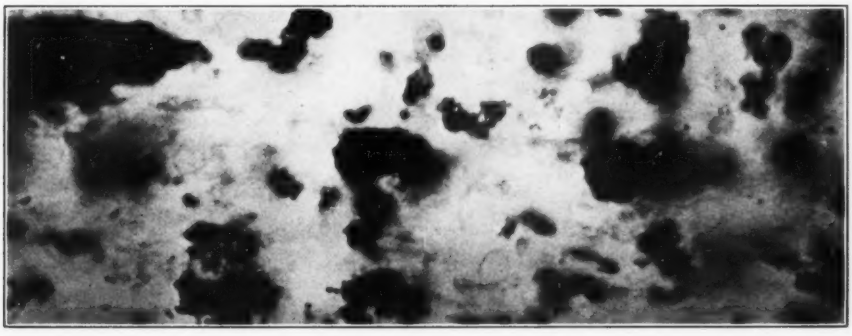

12

A COMPARATIVE HISTOLOGICAL STUDY OF ACUTE MENINGO-ENCEPHALITIS PRODUCED IN RABBITS BY THE VIRUSES OF NEUROVACCINIA AND HERPES SIMPLEX *

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The purpose of this investigation was to make an attempt to compare the histological picture of neurovaccinal meningo-encephalitis in rabbits with that produced by the herpes virus, making allowance for the many apparently pathological changes which are found in the brains of uninoculated laboratory rabbits.

Levaditi, Harvier and Nicolau [1] in 1922, and more recently Turnbull and McIntosh,[2, 3] have published accounts of the histology of the neurovaccinal disease in which they agree that the major pathological changes are found in the meninges. Levaditi and Nicolau [4] describe a mononuclear infiltration of the pia, preceded in the first two days after inoculation by a polymorphonuclear infiltration. They describe the meningeal vessels as surrounded by mononuclear cuffs, the condition being very marked in the septa. According to them, the initial lesion is a true vaccinal pustule in the dura mater. No account of inclusion bodies in neurovaccinal encephalitis has been found.

Da Fano [5] in 1923 described the general picture of herpetic meningo-encephalitis in rabbits with great thoroughness, but he did not then give a very clear account of the intranuclear inclusions. These had been noted by several workers, Levaditi *et al.*,[1, 6] Luger and Lauda, [7, 8] and especially Lipschütz.[9, 10, 11] Further studies on this subject have been made by Goodpasture,[12, 13, 14] and by Cowdry,[15] and a summary of the work up to 1928 is given in Rivers' "Filterable Viruses" by Cowdry.[16]

The variety of apparently pathological lesions which are seen in the brains of "normal" uninoculated laboratory rabbits is notorious. They may be classified roughly as follows:

1. Round cell infiltrations unassociated with any inflammatory or necrotic changes or with any demonstrable parasite. These occur

* Received for publication May 16, 1930.

beneath the ependyma, where it is possible that they represent a normal reservoir of young microglia cells (Penfield[17]). They are also seen in isolated scattered collections through the brain substance. Although it is impossible to demonstrate the presence of a parasite, it is equally impossible to say that these lesions are not either recent or healed lesions of parasitic origin.

2. The parasitic disease described by Wright and Craighead,[18] Levaditi *et al.*,[19] Twort and Archer,[20] Bender[21] and McCartney.[22] The lesions of this disease consist of round cell infiltrations of the meninges, perivascular cuffs and collections of cells beside vessels, necrotic areas in which the parasite may be seen, and cysts containing the "spores" of the organism. In the present series, lesions of these types were frequently met, but in only one brain (that of a rabbit which died of an apparently typical herpetic encephalitis on the seventh day after inoculation) was it possible to demonstrate the parasite itself.

3. Holes and spaces, occasionally with a small nucleus on their extreme edge, containing a hyaline material which stains with mucicarmine and with carbol fuchsin-formaldehyde. In the series under consideration, this picture occurred in two brains, one uninoculated, the other neurovaccinal. But in nearly all of them, spaces were seen which differed only in that they were empty. In brains fixed in formalin, the above description holds exactly. In Zenker-fixed material, however, the holes were smaller and less regular, and what mucoid staining there was was diffuse. This finding is included for the sake of completeness and because of its resemblance to the mucoid degeneration of Buscaino which has been discussed by many writers, including Ferraro.[23] It was not correlated with any other changes, and in view of the action of formalin on cerebral lipoids (Weil[24]), the extent of its pathological significance is not clear.

Material and Technique

Neurovaccinia: The strain of vaccinia used was one derived from calf lymph and "adapted" to the rabbit brain by Dr. Fei-fang Tang by testicular passage. I was enabled to use it through the kindness of Dr. Ward.

Six "normal" rabbits were injected intracerebrally with 0.5 cc. of a 5 per cent triturate in saline or hormone broth of the brains of

rabbits that had died of the disease. Injections were made under light ether anesthesia, the skin and bone over the frontal lobe being pierced with a heated iron point, and the injection being made directly into the brain. In order to avoid mechanical injury to the brain, two other rabbits received similar injections into the cysterna magna, between the atlas vertebra and the occiput.

The typical course of the disease was as follows: The temperature rose on the second or third day to between 104° F and 106° F, falling rapidly to below normal shortly before death. The animals became weak and thin, and sometimes tremulous. No fits or definite paralyses were observed, except in one of the cysterna rabbits which developed a flaccid paralysis of the right fore limb immediately after the operation. Shortly before death most of the animals developed head retraction: they died with the head drawn right back and the back arched by a vigorous contraction of the spinal muscles, thus calling to mind the basal meningitis of man.

Death occurred on the third day in two rabbits, and on the fourth day in the other four rabbits. From time to time the brains of control rabbits that had received similar injections were ground up and injected intradermally into the shaved skin of other rabbits. Typical skin lesions were produced with dilutions of 1:1000 of the 5 per cent suspension.

Herpes: Brains of four rabbits treated as above, but with herpetic material, were examined. Of these, two died on the fourth day, one on the fifth and one on the seventh. All four showed the classical symptoms of herpetic encephalitis: salivation, teeth grinding, hyperexcitability and epileptiform fits, etc., — a condition which contrasts strongly with the neurovaccinal disease, in which the meningitic side of the picture predominates.

Brains were removed immediately after death, except in one instance, when the rabbit died in the night. Animals which were obviously moribund (*i. e.*, with head retraction, subnormal temperature, etc.), were etherized.

After removal, brains and cords were placed immediately in Zenker's fluid (with acetic acid) or in isotonic formol saline (10 per cent formaldehyde, neutralized with MgO). After one or two hours of preliminary hardening, they were cut in slices and returned to the fixative. Transverse paraffin sections from an average of seven planes were examined. All were stained with hematoxylin and eosin,

Giemsa, Mallory's phosphotungstic acid hematoxylin and Mayer's mucicarmine, and with carbol fuchsin one-fourth strength decolorized with strong formalin (see Wright and Craighead [18]). Most of the sections were also stained with Weigert's fibrin stain, in search of organisms.

Frozen sections, 15 microns thick, from the brains that were fixed in formalin were stained by Spielmeyer's iron hematoxylin method to show changes in the myelin sheaths.

Macroscopic Changes

The degree of vascular engorgement of the meninges differed more between the different brains of one disease than between the two diseases in general. In three of the brains small hemorrhagic lesions were visible at the site of injection.

Microscopic Examination

The Meninges: In both diseases a marked meningitis was present in all cases, extending over any part of the brain and cord, and in the pial infoldings and septa. A perivascular arrangement of the cellular elements was often evident, but the greater part of the infiltration was spread diffusely through the pia-arachnoid.

Vasodilatation and hemorrhage were marked over the cerebellum. In three such hemorrhages (two vaccinia and one herpes) which had broken into the cerebellum, reaching the granular layer, there were masses of tissue containing all the elements of bone marrow (blood cells of all kinds, eosinophiles, large vacuolated fat cells, giant cells and many mitoses).

In 50 per cent of the vaccinia cases there were patches of nuclear degeneration scattered through the meninges, recalling Levaditi's statement that a true vaccinal pustule forms. This finding occurred rarely in the herpetic brains.

The relative amounts of fibrinous exudate and cellular reaction varied in different brains, and in different parts of the same brain. The predominant cell was a small round cell, probably a lymphocyte. In all cases, but in the vaccinal much more than in the herpetic, there were large numbers of polymorphonuclear cells with eosinophilic granules. Occasional plasma cells were seen, but there were not nearly so many as in more chronic cases of herpes.

In both diseases there were many larger cells with pale, round or oval nuclei, which from their frequent relation to blood vessels were probably of endothelial origin. In both diseases there were similar patches of submeningeal infiltration with round cells, the meningitic process extending into these areas in the form of perivascular cuffs, and the cytoplasmic processes of glia cells staining with basic dyes.

In brief, the herpetic meningitis resembles that of neurovaccinia in its essential mononuclear cytology and in its distribution, but it is consistently less severe, the meningeal vessels are not so much dilated, and there are fewer polymorphonuclear cells. Moreover, in the herpetic brains, inclusion bodies may be seen in all types of cells except polymorphonuclears, but they were never seen in the vaccinal cases.

Blood Vessels: Vasodilatation was more marked in both meninges and brain substance in the neurovaccinal cases than in the herpetic. In the vaccinal brains an occasional moderate dilatation was seen in such places as the cortex, the base of the midbrain, the corpora quadrigemina and the thalamus. In the cortex, the smallest vessels were the most affected and often contained eosinophilic leucocytes.

Cuffs were seen in all the herpetic brains, and in all except one of the vaccinal. They were more plentiful in the herpetic. They may be divided into two classes:

(1) Submeningeal, *i.e.*, on vessels running in from patches of infiltrated meninges. In "normal" control rabbits the straight vessels perpendicular to the surface are plentifully supplied with nuclei, some endothelial, some of the small round cells. This was a constant finding in the diseased brains, but it was often possible to say definitely that the numbers of both types of cell were increased. Nuclear degeneration (pyknosis) and eosinophiles were not uncommon in the walls of these vessels, with or without a definite increase in the number of cellular elements. These cuffs probably represent direct spread of the meningitic process along the Virchow-Robin space.

(2) Isolated cuffs were seen in various parts of the brain, well removed from the meninges, but it was found impossible to differentiate between such cuffs when they are due to the virus activity, and when they are an expression of spontaneous disease. They were particularly numerous in the one herpetic brain in which the parasite of the spontaneous encephalitis was found.

These scattered cuffs fall roughly into two subdivisions, those that surround the vessel completely, and those that lie to one side of it. The latter kind were rather more common in the vaccinal brains, but both occur in uninoculated brains, including those of four rabbits fourteen days old, which had shown no symptoms of disease during life. In the vaccinal brains small perivascular hemorrhages and occasional perivascular deposits of hyaline fibrinous material suggest a scattered local damage to the walls of the small vessels, an observation which may have significance in view of McIntosh and Scarff's statement [25] that in generalized vaccinia, the lesions are essentially in the vascular endothelium.

Areas of perivascular softening, such as are described in various human encephalitides, were not determined.

Pigment: Pigment was noted in only one brain, in the pia mater.

Edema: Empty spaces around cells and vessels, and in the fiber tracts were common in all brains.

Local Lesions: Injection lesions take the form of areas of hemorrhagic necrosis, containing mononuclear cells and surrounded by diffuse infiltration with eosinophiles and glia cells. In the vaccinal brains no particular changes were noted in the neurones near these lesions, but it is true that in the six "intracerebral" brains what neuronic change there was lay further forward in the brain than it did in the two "cysterna" brains. In the herpes brains neighboring changes in the neurones were more marked, and inclusion bodies were commonly found.

Focal collections of cells, whether round cells or glia, occurred in brains of both series, but as similar collections with or without signs of necrosis occurred in five uninoculated control rabbits, no special significance can be attached to them. One such lesion in one of the herpetic brains was accompanied by many inclusion bodies, but this fact alone is not sufficient to prove that the whole lesion was herpetic in origin.

In one vaccinia brain there was a large hemorrhage into the third ventricle, and in another into the external capsule, but both of these were probably caused by the injection injury.

Changes in Nerve Cells: There was considerable variation in the amount of change in different brains, but in most of them there was a slight degree of all the changes mentioned below. The herpetic brains showed more change than did the neurovaccinal.

In some of the brains neurone damage was scattered throughout the brain, isolated cells being affected, but in others there were areas in which many of the cells present suffered. Such areas also showed vasodilatation and glial increase.

Unlike the pathological changes in the glia, neuronic changes do not necessarily underlie areas of intense meningitis, nor can their presence or distribution be correlated with the injection injury, save in the broadest way. Changes, other than inclusions, noted in the neurones were:

Nuclear swelling, which was the commonest. It was particularly prominent in the herpes brains.

Chromatolysis, in all stages, from peripheral condensation of dark Nissl granules to their total disappearance.

Neuronophagia, the neurone being in varying stages of disintegration, with the glia nuclei inextricably mixed in its substance. This type of degeneration was rare, but it did occur; Levaditi comments on its absence in neurovaccinia in rabbits. It is not possible to say that its presence in such animals in this series was not due to one of the spontaneous changes already referred to.

Pseudoneuronophagia, which, as Da Fano and Ingleby [26] recall, may be distinguished from the true variety by the state of the neurone and the presence of an intact boundary between it and the glia cells.

Satellitosis, an increase in the number of satellite cells; an unreliable sign.

Eosinophilic Degeneration: The remains of degenerated neurones stain red with eosin. They may be isolated or surrounded by phagocytic microglia. Some nuclei stain, in whole or in part, a purple-red with dyes containing eosin. Their cytoplasm is sometimes shrunken or overstained with methylene blue. This condition is observed in most of the brains and in uninoculated controls. It is to be distinguished from the oxychromatic degeneration of Luger and Lauda,[27] which they describe as the first stage in the formation of herpetic inclusion bodies. The distinction is easily made, for in the Luger and Lauda type, the ground substance of the nucleus stains a deeper pink and has a ground-glass appearance, the chromatin is exaggerated and arranged peripherally, and the cytoplasm is unaffected. Both types are found in the herpetic brains.

Vacuolation: Neurones with vacuolated cytoplasm are found mainly in those areas that are badly affected with neuronophagia, etc.

In summarizing these neuronic changes, emphasis must be laid on their infrequent occurrence in the vaccinal brains. The vast majority of the neurones in any one brain show no abnormality.

Inclusion Bodies: These constitute the one distinctive feature of the herpetic disease. No inclusion bodies, either intranuclear or cytoplasmic, were seen in any of the vaccinal brains, nor has reference to them been found in the literature. In the herpetic brains they occurred every time. Their appearance coincided with the descriptions of them given by other observers. They were frequently multiple and often accompanied by a fine eosinophilic dust, not unlike that seen in the nuclei of neurones in other conditions; but the "myelin-like" bodies described by Goodpasture [13] were not seen, even though the brains were injected with Zenker's fluid via the carotids while the animal still lived under the anesthetic.

Inclusion bodies were found with considerable constancy in the Ammon horn, where they were accompanied by both types of oxychromatic change and an irregular proliferation of glia cells.

Neuroglia: The most marked proliferation of glia occurred beneath the worst patches of meningitis. In these places it was common for the cytoplasmic processes of the cells to stain with methylene blue. In the fiber tracts there was occasional evidence of oligodendroglia proliferation, but this was also seen in some of the uninoculated rabbits. Areas in which there was marked neuronic change also showed some glial increase. Occasionally a glia cell with a small shrunken nucleus and vacuolated cytoplasm, giving an appearance similar to that described by Penfield and Cone [28] as acute swelling of oligodendroglia, was encountered, but the condition was not common.

In the herpetic brains inclusion bodies were not uncommonly seen in glial nuclei. Collections of glia cells into unrelated clumps, with or without signs of necrosis, and subependymal gliosis have already been referred to in connection with the spontaneous lesions. It is difficult to assign any specific meaning to them. Gitterzellen were observed in two brains, in each case just below the acutely inflamed meninges. In material fixed in Zenker's fluid, the vacuoles stained pink and were larger; in formalin sections they were smaller and colorless when stained with Giemsa.

Changes in the Myelin Sheaths: Demyelination not unlike that occurring in multiple sclerosis has been noted by Turnbull and McIntosh [2] in postvaccinal encephalitis in man, by McIntosh [3] in encephalitis following smallpox, and by Perdrau [29] in chronic encephalomyelitis in dogs. In these neurovaccinia and herpetic brains a search was made for similar changes by cutting thick frozen sections of formalin-fixed material and staining with Spielmeyer's iron hematoxylin method.[30]

No extensive perivascular, or other destruction of the myelin sheaths at all comparable to that described by the above workers, was found in these acute brains, but scattered diffusely through all the inoculated brains examined were areas in which some of the sheaths were ballooned in a curious and irregular manner. In the majority of the brains these changes were very slight and rare.

Other appearances encountered in these brains as well as in those of the uninoculated controls were small lateral buds on otherwise normal sheaths, an appearance of beading caused by local kinks and bends, occasional small regular dilatations containing small, highly refractile granules, and a few very thin fibers which stained a uniform black.

Discussion

The findings in this series do not readily lend themselves to any useful generalizations on the pathology of the two diseases studied. The one cardinal point of difference between herpetic meningoencephalitis and the vaccinal disease is the occurrence of intranuclear inclusion bodies.

Both clinically and histologically the meningitic side of the picture is emphasized in the vaccinal disease, but though greater in extent, it is essentially the same in cytology and distribution as in that of herpes.

Other lesions, perivascular cuffs, scattered neuronic and glial changes, and the mucoid degeneration described, cannot be differentiated from similar lesions of "spontaneous" nature; from this series it is not possible to state that any of them are due to the herpetic or vaccinal viruses alone.

The changes in the myelin sheaths are rare and inconstant, and of obscure significance.

The chronological sequence of events, as judged from these brains and from those of rabbits killed in earlier stages of the two diseases is roughly as follows: The first meningitic change is an infiltration with polymorphonuclear leucocytes, accompanied by some fibrinous exudation. By the fourth day, at which time most of the neurovaccinal animals die, the mononuclear invasion is at its height, but polynuclear cells are still present in considerable numbers. By the sixth day, which represents the time of death of most of the herpetic animals, polynuclears are scarce. Plasma cells are seen occasionally on the third and fourth day, but they become more common at a later date, especially in "chronic" herpes cases, which live to the eleventh or twelfth day. Perivascular cuffs appear early in both diseases, but their origin may be independent of either virus.

Summary

1. The histological findings in six neurovaccinal and four herpetic brains are described.

2. Intranuclear inclusion bodies are found to be the only sure distinctive feature of the herpetic disease. In other respects the two diseases are essentially similar.

3. In neurovaccinia, the meningitis is the most conspicuous finding, both clinically and histologically.

4. Various spontaneous lesions in the brains of uninoculated laboratory rabbits are discussed.

5. Myelin sheath changes are described in the brains of herpetic and vaccinal animals, but perivascular demyelination of the kind characteristic of postvaccinal encephalitis in man was not seen. It is possible that the duration of the disease is a factor in the development of such a condition.

Since preparing this paper for publication, valuable articles on the histology of neurovaccinal encephalitis in monkeys and rabbits by Hurst and Fairbrother and by McIntosh and Scarff, have appeared in the *Journal of Pathology and Bacteriology* (1930, **33**, 463 and 483). These papers are in agreement on the cardinal points of histology, such as the absence of inclusion bodies and the accentuation of the meningitis. In neurovaccinia, McIntosh and Scarff emphasize the rôle of the vascular endothelium; this is undoubtedly damaged in the smaller vessels and there are occasional evidences of its pro-

liferation, but from the preparations in this investigation the impression is derived that this feature of the disease is subsidiary to the general inflammation, and, moreover, it seems to be just as much a feature of herpetic encephalitis as it is of neurovaccinal.

I wish to thank Dr. Hans Zinsser and Dr. Hugh K. Ward for constant advice and help, Dr. Raymond Morrison for help with many questions of neuropathological import and assistance in staining technique, and Mr. C. V. Seastone, Jr., for the care he has taken with the photomicrographs.

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DESCRIPTION OF PLATES

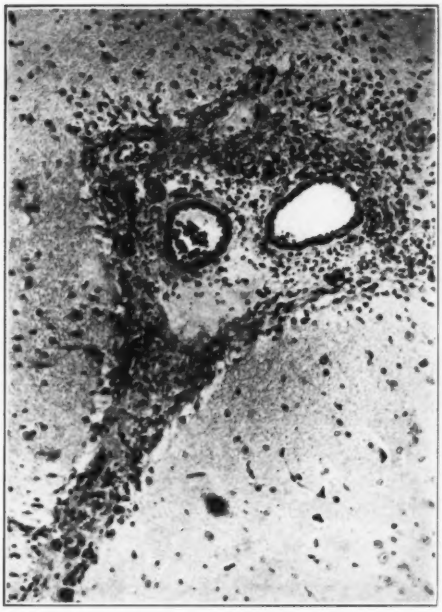
PLATE 143

FIG. 1. Neurovaccinal meningitis in one of the pial septa, showing perivascular arrangement of the mononuclear cells. Formalin fixation. Hematoxylin and eosin stain. × 125.

FIG. 2. Extension of vaccinal meningitis along Virchow-Robin space. Formalin fixation. Hematoxylin and eosin stain. × 125.

FIG. 3. Portion of Fig. 1. × 600.

FIG. 4. Herpetic meningitis. Zenker's fixative. Giemsa stain. × 125.



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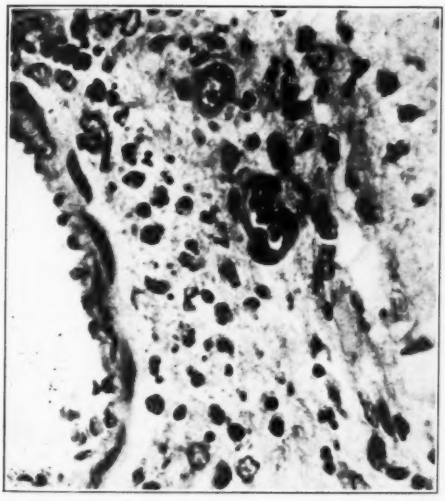

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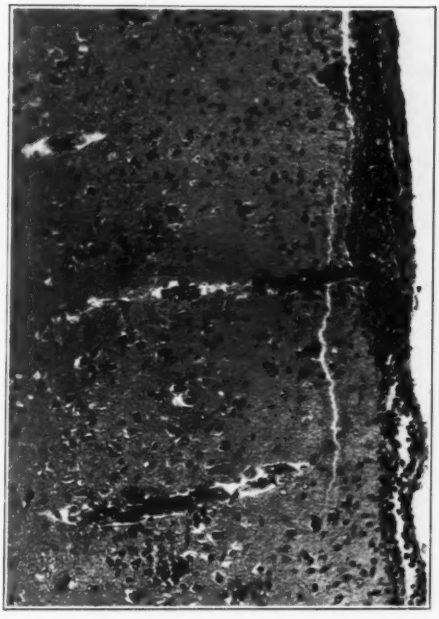

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PLATE 144

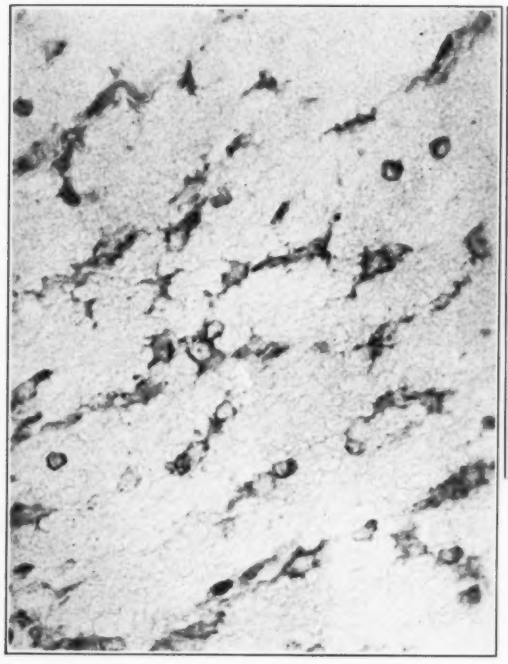
FIG. 5. Submeningeal gliosis in neurovaccinal brain. The cytoplasm of the glia cells has stained. Formalin fixation. Giemsa stain. × 600.

FIG. 6. "Mucoid" change in a vaccinal brain. Formalin fixation. Mallory's phosphotungstic acid hematoxylin and Mayer's mucicarmine stain. (The gray, homogeneous areas stained a bright pink.) × 600.

FIG. 7. Neurovaccinia. Vacuolated neurones. Formalin fixation. Giemsa stain. × 600.

FIG. 8. Ballooning of myelin sheaths. Spielmeyer's iron hematoxylin method. × 1250.

FIG. 9. Ballooning of myelin sheaths. × 600.



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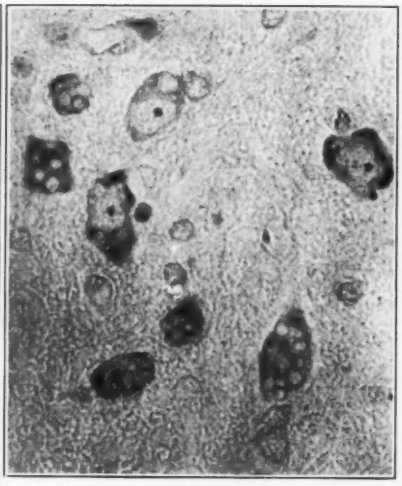

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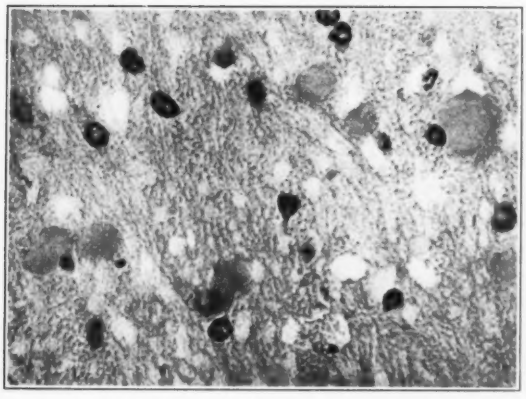

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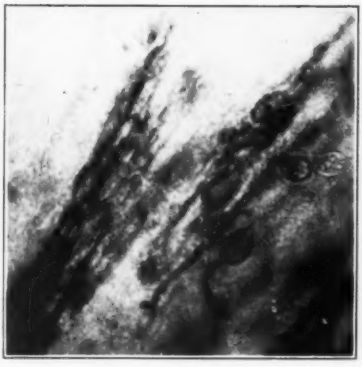

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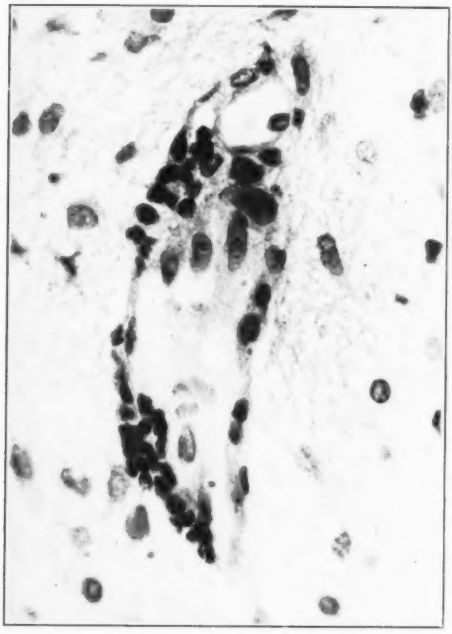
PLATE 145

FIG. 10. Nodular cuff. Vaccinal brain. × 600.

FIG. 11. Lesion containing the parasite of spontaneous encephalitis. Formalin fixation. Carbol fuchsin and methylene blue stain. × 125.

FIG. 12. Perivascular lesion in 14 day-old "normal" rabbit. Zenker's fixative. Giemsa stain. × 600.

FIG. 13. Parasite of spontaneous encephalitis. Carbol fuchsin stain. × 1250.



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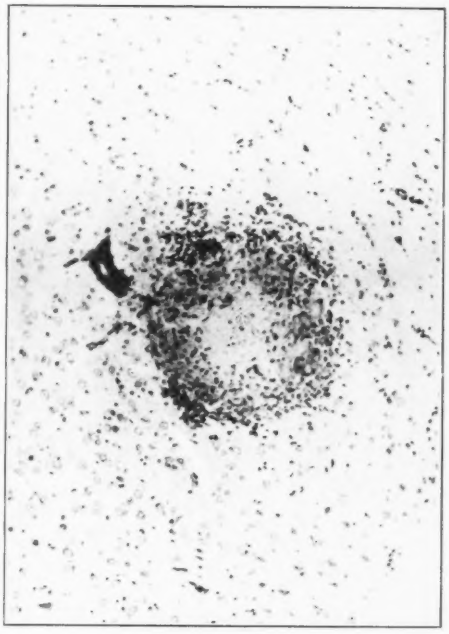

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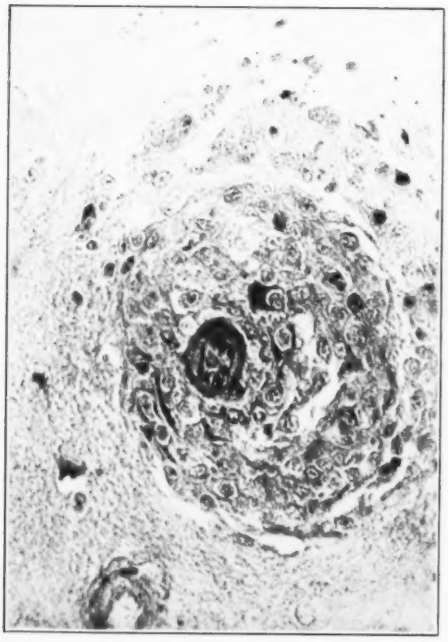

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